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Award Number:
W81XWH-10-1-0534

TITLE:
Undiagnosed Small-Fiber Polyneuropathy: Is it a Component of Gulf War Illness?

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REPORT DATE:
September, 2014

TYPE OF REPORT:
Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
September 2014	Final Report	1 July 2010 – 30 June 2014

4. TITLE AND SUBTITLE
Undiagnosed Small-Fiber Polyneuropathy: Is it a Component of Gulf War Illness?

5a. CONTRACT NUMBER

5b. GRANT NUMBER W81XWH-10-1-0534

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Boston, Massachusetts 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES
U.S. Army Medical Research
and Materiel Command

Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S REPORT NUMBER(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The term small-fiber polyneuropathy (SFPN) refers to body-wide dysfunction/degeneration of small-diameter axons that transmit pain and control the body's autonomic functions. The vague, widespread symptoms of SFPN overlap with those of Gulf War Illness (GWI). We propose that there may be a SFPN component to GWI. To diagnose SFPN in Gulf War-ill veterans, we tested normal control subjects and patients with definite SFPN to compare the sensitivity and specificity of the best known tests. We also tested Gulf War veterans with and without GWI to identify how often this neurological illness is masquerading as GWI, and we applied the same tests (autonomic function test, skin biopsy, and neurological exam) to subjects diagnosed with fibromyalgia (FM). We also characterized our subjects' health with a series of validated questionnaires. By doing so, we found markers of SFPN in Gulf War veterans and FM patients in approximately the same ratio.

15. SUBJECT TERMS
Gulf War Illness, small-fiber polyneuropathy, SFPN, neurite, skin biopsy, autonomic function test, AFT, axon flare, fibromyalgia, chronic multisymptom illness

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	53	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

The term small-fiber polyneuropathy (SFPN) refers to body-wide dysfunction and degeneration of the small-diameter axons that transmit pain and control the body's autonomic (involuntary) functions. The vague, widespread symptoms of SFPN overlap substantially with those of Gulf War Illness (GWI). SFPN is hard to diagnose clinically and requires special tests. We propose that there is a SFPN component to Gulf War Illness. To identify and apply the best tests to diagnose SFPN in Gulf War-ill veterans, we recruited, screened, and tested normal control subjects and patients with definite SFPN to compare the sensitivity and specificity of the best current tests (skin biopsy and comprehensive autonomic-function testing (AFT)), as well as a potential new test (axon flare reflexes to vasodilators) (Aim I). We applied the best of these tests to compare results in Gulf War veterans with and without Gulf War illness to identify how often this diagnosable and treatable neurological illness is masquerading as Gulf War Illness, and we applied the same tests to subjects diagnosed with fibromyalgia (Aim II). By doing so, we not only established a relationship between symptoms of Gulf War Illness and SFPN, and between SFPN and fibromyalgia, but also determined which tests are the most diagnostically useful and should be adopted for more widespread clinical use. This report summarizes the findings of Tasks 1, 2, and 3 of Specific Aims I and II of the basic statement of work which is included at Appendix 1, and presents clinical evidence of small-fiber polyneuropathy in patients previously diagnosed with fibromyalgia and in juveniles.

SUMMARY OF PROGRESS AGAINST SPECIFIC AIMS:

The start of this study was dedicated to establishing normative neurite densities from the general normal population and identifying the test(s) of greatest utility in diagnosing small-fiber polyneuropathy, which had been identified as a strong need in the neurology community [1]. After making a statistical determination of the best tests, we recruited and tested Gulf War veterans with those tests. Please refer to the Statement of Work, included at Appendix 1, for the complete study plan. During Year 3 of this study we requested and were granted a one-year extension without funds (EWOF) to compensate for slow recruitment of veterans. Also, during the first quarter of Year 3 we added a concurrent study of small-fiber polyneuropathy among fibromyalgia patients to Task 3 of Specific Aim II because of its relevance to the symptoms and diagnoses often sought and provided to Gulf War veterans, including those participating in this study. The results of that study are discussed with the outcomes of Task 3.

Specific Aim I. To determine which specific measurements of skin innervation, autonomic function, and skin blood flow provide the most sensitive, specific, and practical objective test for SFPN.

Task 1. Establish demographically correct skin biopsy norms. A cohort of 120 normal controls will be established to provide the necessary range of ages, sexes and ethnicities

a. Recruit, screen and test 120 normal controls. Some subjects have already been studied to provide preliminary data for this application. (months 1 – 6)

b. Multivariate data analysis to determine which of the three demographic variables tested (age, sex, race/ethnicity) influences the normal values for density of skin innervation and to generate the norms and limits between the normal and abnormal ranges necessary for clinical diagnostic use. (months 6 – 8)

c. To prepare and publish a manuscript in a high-impact neurological journal that will make these norms available for medical use world-side. An internet version will also be made available. (months 8 – 20)

Task 1 involved establishing demographically normal values for the density of innervation in the distal-leg skin biopsies used to diagnose small-fiber polyneuropathy. Accurate diagnosis of disease depends entirely on accurate definitions of the normal range. Early studies of skin neurite quantification by skin biopsy [2] established a cutoff value below which a neurite density was considered abnormal regardless of age, gender, or ethnicity. This was considered the standard of diagnosis until more recent work which has begun to show evidence of differences in neurite density by age and gender [3]. A major shortcoming of these studies, including a world-wide collaboration of neurite density databases [4], is that the study populations were either not diverse or they did not identify ethnic differences in neurite density in addition to gender differences. Our study expanded on these findings by studying a cohort of at least 120 normal control subjects of various ages, genders, and ethnicities.

Methods: Task 1a: Recruitment. We recruited normal subjects primarily through in-house advertisement and through the Research Study Volunteer Program (RSVP for Health) administered by our Clinical Research Program. All normal subjects were initially telephone-screened to rule out confounding health issues. In addition, normal subjects first underwent a 2-hour fasting glucose-tolerance test (GTT) to rule out occult diabetes, which is increasingly prevalent and carries high risk of polyneuropathy.

Methods: Task 1a: Skin biopsy. Skin biopsies were performed in our JCAHO-accredited laboratory. After informed consent, a site (10 cm above the ankle) was anesthetized and one or two 2- or 3mm diameter skin punches were removed using sterile technique and the site covered with a Band-Aid. Tissue samples were immediately fixed and then sectioned (using a freezing sliding microtome) and processed using standard methods. For PGP9.5-IR, Zamboni's fixative, 50 micron sections, and a 1:1200 dilution of polyclonal antibody specific for the pan-axonal enzyme ubiquitin hydrolase (PGP9.5) were used, followed by standard DAB labeling. For each biopsy, a single morphometrist then counted the total number of separate immuno-positive neurites within the entire epidermis which was then normalized to the skin surface area to yield a neurite density per square millimeter of skin surface.

Outcomes: Task 1a: We had already studied the 120 normal controls during Year 1 of this study but continued to add to that total because (1) we were able to pool results of prior studies in our laboratory (using identical techniques) with those obtained under this study and (2) we also had to recruit additional controls because we needed additional subjects who could undergo the complete set of tests for Task 2 (glucose tolerance test, AFT, skin biopsy, axon flare), and many of the subjects who were studied previously were not available to complete all required Task 2 study tests. By the end of calendar year 2011 we had studied 215 subjects aged 18 and older.

Our actual number of subjects studied was greater, but we removed 14 subjects from the analysis either for pre-existing conditions that were not disclosed during the initial screening, or once it was demonstrated by 2-hour fasting glucose tolerance test that they had impaired glucose tolerance (i.e., pre-diabetes), as defined by criteria of the American Diabetes Association. The age distribution of the included subjects is summarized in Table 1.

Age Range	No.
18-19	18
20-29	56
30-39	38
40-49	33
50-59	26
60-69	26
70-79	12
80-89	6
Total	215

Table 1. Distribution of normal adult subject ages

In addition to the adult normal controls listed in Table 1, we biopsied 25 youngsters aged 14-17 through other studies, for a total of 240 subjects with which to construct the normative series. Although pediatric norms were outside of the scope of this study, the biopsy results of the youngsters anchored the lower end of the normal biopsy curve from which the multivariate analysis was derived. Thus, their biopsies remained part of the data set for the analysis of this study even though they were not studied under this grant. For the purpose of advancing the analysis of Task 1b, we discontinued adding to the normal dataset as of the end of calendar year 2011, even though more biopsies were collected through the rest of Year 2 of this study. Details of the genders, and ethnic and racial composition of the full data set is shown on Table 2. The full data set is presented graphically in Figure 1 where the youngsters (under age 18) are in red.

Ethnic populations	Male	Female
White	80	105
Asian	17	11
Indian subcontinent	4	0
Black	5	10
Hispanic	5	1
Pacific Islander	1	1
Non-Asian	91	117
All Asians	21	11
Total	240	

Table 2. Ethnic populations in the skin biopsy cohort. These totals include young subjects aged 14-17.

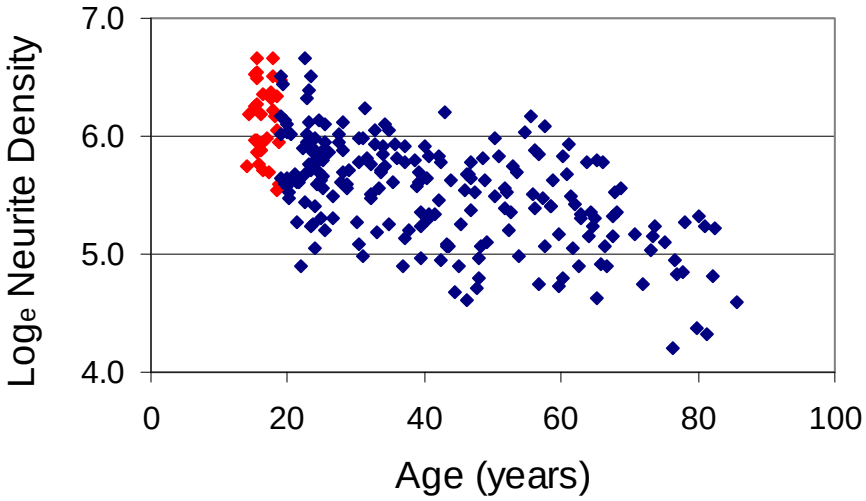

Figure 1. Logarithmic plot of neurite densities of 240 normal controls. Subjects under age 18 (not part of this study, but included in the analysis) are in red

Methods: Task 1b: To demonstrate the necessity of a multivariate regression analysis of our normative skin biopsy series, we looked for significant differences in neurite density among the populations we studied. We then engaged biostatistician Dianne Finkelstein, PhD, who directs MGH's Biostatistics Unit to review our findings and to develop the final multivariate regression fit of our results to establish normative skin biopsy values.

Outcomes: Task 1b: We confirmed that among our normal controls, Asian subjects had significantly higher neurite densities than non-Asian subjects. We found that there was also a small but significant difference between Black and non-Black subjects, but our population was insufficiently powered in Hispanic/Latino, or Hawaiian/Pacific Islanders to show any significant differences involving those populations. We also observed that females in general had significantly higher neurite densities than males. The Asian composition by gender of the final normal cohort is presented graphically in Figure 2. Note that a semi-log plot provided the best statistical fit.

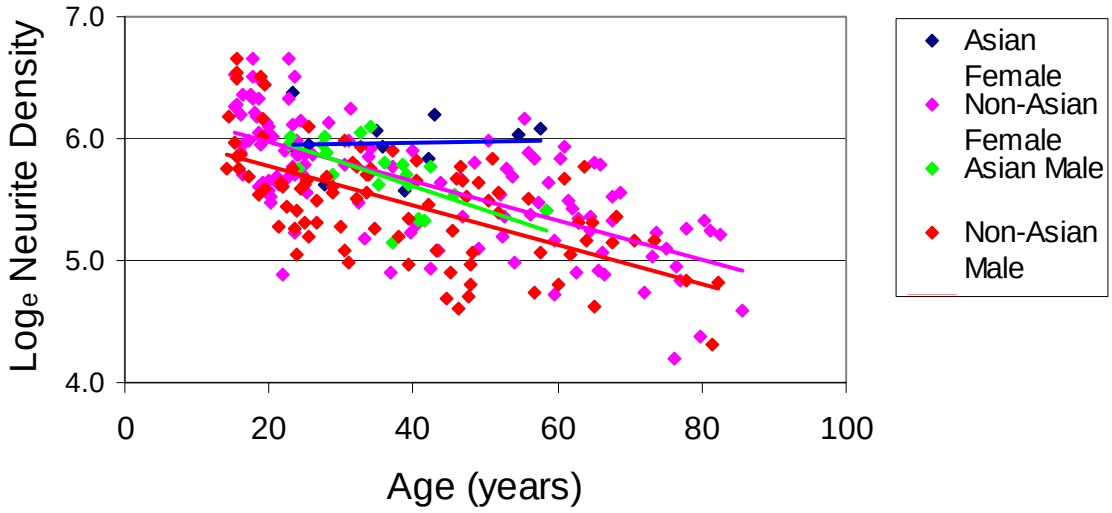

Figure 2. Asian and non-Asian composition of the normal control cohort for skin biopsy

We compared neurite densities among Asian and non-Asian populations within an age-matched group. The statistical results are summarized in Table 3, and indicate that there is a significant difference in neurite density between Asians and non-Asians ($p=0.0032$) while the age distribution among the two groups was not significantly different ($p=0.3479$).

Neurite density	n	avg enf	std dev	std error
Asian	32	341	94	16.6
Non-Asian	106	276	115	11.1

t test enf: p = 0.0032
(significant difference in neurite density between the two populations)

Ages	n	avg age	std dev	std error
Asian	32	35.94	9.69	1.7
Non-Asian	106	37.86	11.23	1.1

t test ages: p = 0.3479
(no significant difference in the ages of the two populations)

Table 3. Comparison of epidermal nerve fiber (enf) density and ages for Asian and non-Asian subjects in a specific age range [age range: 22.84 to 58.34 years (Asian), 22.69 to 58.54 years (non-Asian)]

We also compared neurite densities among females and males of all ethnicities over the entire range of ages. The results are summarized in Table 4 and indicate that there is a significant difference in neurite densities between females and males (p = 0.0084), while the age distribution among the two groups was not significantly different (p=0.265).

Neurite density	n	avg enf	std dev	std error
Female	128	323	150	13.3
Male	112	276	127	12.0

t test enf: p = 0.0084
(significant difference in neurite density between the two populations)

Ages	n	avg age	std dev	std error
Female	128	40.49	20.8	1.84
Male	112	37.79	16.5	1.56

t test ages: p = 0.265
(no significant difference in the ages of the two populations)

Table 4. Comparison of epidermal nerve fiber (enf) density and ages for Females vs. Males over the entire study age range [age range: 15.27 to 85.71 years (Female), 14.00 to 82.26 years (Male)]

Having shown that there are significant differences in neurite density among several of the populations we studied, we also observed that young subjects had a superabundance of neurites that tended to skew that linear regression fit. We concluded that the best semi-log data fit was to

a "bimodal" model with a break point at age 22, i.e., data are analyzed separately for best fit below age 22 and above age 22. The statisticians then fitted a "piecewise" linear regression model for log density as a function of age, gender, and ethnicity, with a change point at age 22. The model fit was confirmed with an analysis of the residuals (for normality and homoscedasticity). The model fit (showing gender differences only) is shown in Figure 3.

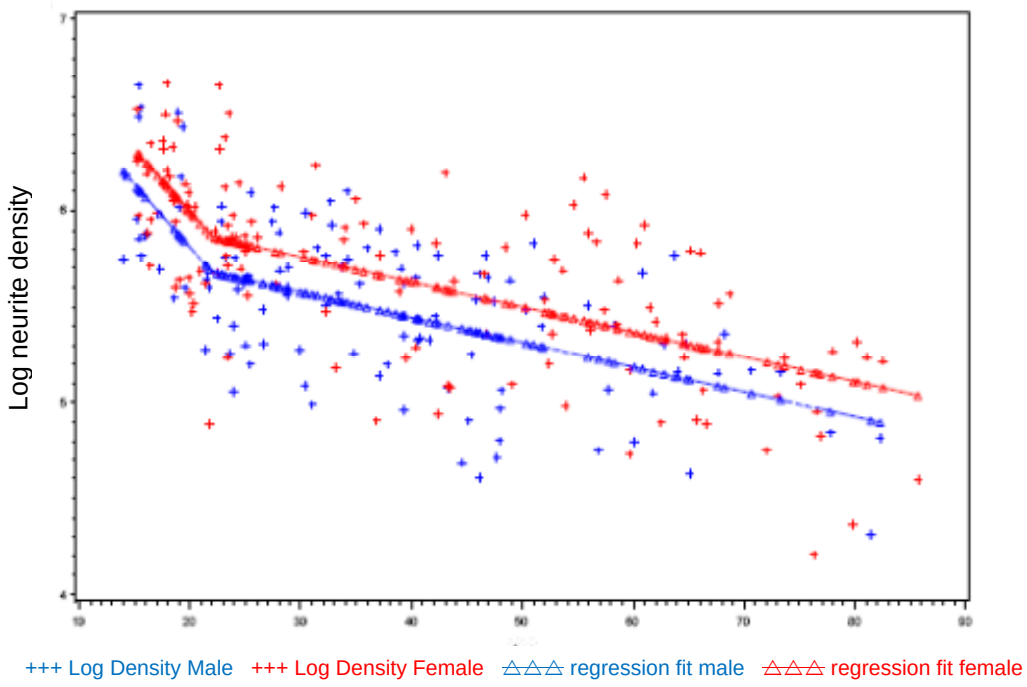

Figure 3. Log_n density vs. age with breakpoint at age 22.
Upper line (red) is female, lower line (blue) is male

Thus a multivariate regression analysis that accounts for the variations attributed to age, gender, and ethnicity with a break point at age 22 is appropriate and is presented in Equation (1). We applied this regression to all subsequent analyses of neurite densities to derive the appropriate percentile rank (see Task 1c).

Equation (1):

$$Log_n (\text{neurite density}) = 7.06 - 0.064(\text{age}) + 0.051(\text{age} - 22) + 0.201(\text{gender}) + 0.253(A) - 0.235(B)$$

Where
neurite density is in neurites/mm^2
age is in years,
gender = 1 if female, 0 if male,
A = 1 if Asian, otherwise = 0,
B = 1 if Black, otherwise = 0

Task 1c. A manuscript is being prepared for publication of the results of Tasks 1a and 1b. Availability of the biostatisticians has been a limiting factor for the analysis that is central to the

manuscript. In the interim, we submitted an abstract based on these findings to the American Neurological Association which was accepted as a *Works in Progress* poster for presentation at their Fall 2014 meeting, October 12 in Baltimore. Previously, preliminary results were presented as a poster at the 2010 ANA Meeting [5], which was also honored as a *Works in Progress* presentation.

To make these results accessible to the scientific and clinical community we also developed a neurite density calculator based on the multivariate regression analysis. We plan to install it on our laboratory web site for use by researchers and diagnosticians who wish to verify a diagnosis of small-fiber neuropathy or obtain a predicted value for patients with specific demographics. A sample neurite density calculator screen is shown in Figure 4. Entering Age, Gender, and Race returns an expected normal neurite density ("Predicted Neurite Density value"). However, if a neurite density (in neurites per mm^2 of skin surface) is already known and is entered ("Enter raw Neurite Density") then a Percentile is returned which compares that neurite density with the normal population of our study. The diagnostician may then interpret normal and abnormal range values based on the percentile. It is common practice to interpret neurite densities at or below the 5th percentile as abnormal and indicative of neuropathy.

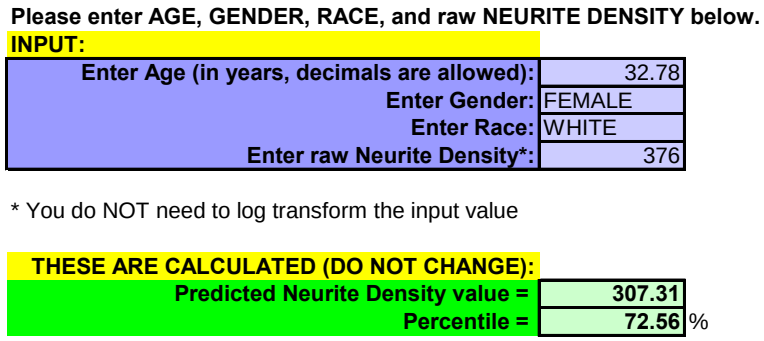

Figure 4. Sample screen shot of the Neurite Density Calculator

Summary of Outcomes for Task 1

We first addressed the need for an evaluation of diagnostic tests for small-fiber polyneuropathy by studying skin neurite density in a cohort of 240 normal controls. We overcame several shortcomings of previous large multi-center collaborations that were homogenous in population [4] by identifying gender and ethnic differences as well as age-related differences in neurite density. We were thus able to establish normative values for a diverse population and, by extension, identify values that were abnormal and indicative of small-fiber polyneuropathy. We developed a neurite density calculator and applied this normative scale to our studies of younger subjects afflicted with widespread chronic pain, veterans, and patients previously diagnosed with fibromyalgia (Task 3). Note that studying younger subjects (which included 15 normal controls) was not funded by this project, but the results are significant and relevant to this study based on the possibility that warfighters of recruitment age may have been affected by similar small-fiber polyneuropathy at a young age before entering service, and this may eventually manifest itself as chronic multisymptom illnesses in adults.

Task 2. Compare the diagnostic sensitivity and specificity of skin biopsy, AFT, and axon-flare measurements to establish best tests for SFPN. Data will be collected from cohorts of 40 screened normal volunteers, SFPN patients, and symptom-matched control patients with severe osteoarthritis.

a. Recruit 40 normal subjects from among the 120 being studied by skin biopsy for Aim I for additional study with AFT and axon-flare measurements. (months 3 – 12)

b. Recruit 40 subjects with definite SFPN from among the several hundred already evaluated for clinical care at Mass. General Hospital by skin biopsy and AFT for additional study of axon-flare measurements. (months 8 – 18)

c. Recruit 40 severe osteoarthritis of the hip or knee from among the thousands such patients followed at Mass. General Hospital for study by skin biopsy, AFT, and axon-flare measurements. (months 8 – 18)

d. Multivariate data analysis to determine which of the tests have greatest potential for clinical diagnostic use. Positive and negative predictive value, diagnostic sensitivity and specificity, invasiveness and cost will be considered. Tests that complement or overlap will be identified. (months 18 – 22)

Task 2 provided the data to establish the best test for SFPN, which has been identified as a diagnostic need [1]. We compared the results of 3 diagnostic tests for SFPN (skin biopsy, AFT, and axon flare) to identify which has the best predictive value. In addition, we ruled out other potential sources of SFPN through telephone screening and 2-hour fasting glucose tolerance test (GTT). While one study indicated a possible correlation between axon flare size, sweat production (one of the AFT tests), and neurite density, it did not include a control comparison group [6]. We are studying osteoarthritis subjects as an added positive control group to demonstrate that although the osteoarthritis subjects are also experiencing chronic pain, as do the SFPN patients, their pain has a non-neuropathic origin, and thus peripheral nerve test results from the osteoarthritis subjects should not differ significantly from the normal controls. To our knowledge this is the first study to use this approach.

Methods: Tasks 2a, 2b, 2c: Recruitment. We recruited subjects mainly through in-house advertisement and through the Research Study Volunteer Program (RSVP for Health) administered by our Clinical Research Program. To the extent possible for normal controls, we invited previously studied subjects to continue in this study. Small-fiber polyneuropathy (SFPN) subjects from the neurology practice at Massachusetts General Hospital were also invited to participate if they received a definite diagnosis of SFPN from a neurologist. We also recruited additional osteoarthritis subjects through their physicians, aided by automated searches through Mass General Hospital's Research Patient Data Registry (RPDR) which is a searchable clinical data repository for patients treated within the Partners Healthcare system (the parent organization of MGH and affiliated hospitals).

Methods: Tasks 2a, 2b, 2c: Autonomic Function Test (AFT). AFT consists of four specific separate tests that are routinely used and endorsed for clinical diagnostic testing for small-fiber

polyneuropathy. AFT was administered by personnel trained by the manufacturer (WR Medical Electronics, Stillwater, MN). The specific tests are (1) QSART (quantitative sudomotor axon reflex test) where sweat production is quantitated from the standard forearm, proximal leg, distal leg, and foot sites in response to iontophoresis of acetylcholine; (2) heart rate response to deep breathing where heart rate variability during inspiration and expiration is measured for at least 5 cycles; (3) beat-to-beat heart rate and blood pressure responses in phases II and IV of the Valsalva maneuver where heart rate variability is measured while the subject is asked to blow into a mouthpiece to sustain a pressure between 40 and 50 mm Hg; and (4) beat-to-beat heart rate and blood pressure responses to tilt where continuous blood pressure and heart rate monitoring is performed for 5 minutes with the subject supine, then the subject is placed in an 80 degrees heads-up tilt position for 10 minutes and subsequently returned to supine for an additional 5 minutes of recording.

Methods: Tasks 2a, 2b, 2c: Axon flare measurement by laser Doppler flowmetry. Laser Doppler measurements of skin blood-flow and axon flare were performed in our laboratory using equipment from Moor Instruments Ltd, Devon, UK. Changes in blood flow rate were measured in response to a vasodilator (histamine or acetylcholine) which was introduced into the skin via iontophoresis. A hollow plastic ring was affixed to a subject's skin which positioned two fiber-optic laser leads, and was also a reservoir for the vasodilator (see Figure 5). After measuring baseline blood flow, a current was applied to the liquid reservoir in the ring to drive the charged vasodilator molecules into the skin. Direct (over the site of iontophoresis) and indirect (in the center of the ring, 3mm distant from the site of iontophoresis) measurements of axon flare intensity due to vasodilation were recorded continuously over time while a laser-Doppler imager provided a sequence of blood flow (flux) maps of the vasodilated region, taken at predetermined intervals. From the blood flow maps, the maximum flare size (in cm) was calculated. Since vasodilation is mediated by small nerve fibers, SFPN would manifest as a reduced flare response.

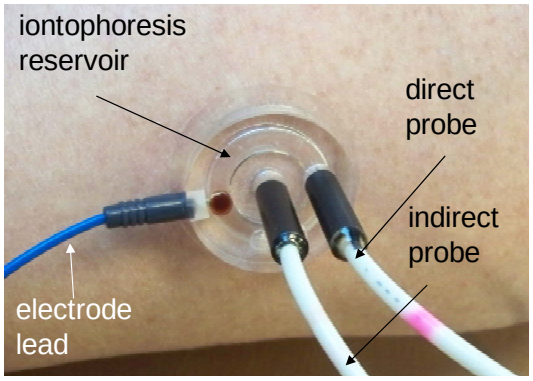

Figure 5. Positioning of the laser Doppler probes for measurement of blood flow. The liquid reservoir holds the chemical that is iontophoresed into the skin by current through the electrode lead.

Methods for skin biopsy were as described in Task 1. In addition, all subjects first underwent a 2-hour fasting glucose-tolerance test (GTT) to rule out occult diabetes, which is increasingly prevalent and carries high risk of polyneuropathy.

Outcomes: Tasks 2a, 2b, and 2c: Table 5 summarizes the recruitment totals of normal, osteoarthritis, and SFPN subjects under Task 2. All subjects were studied with each of the 4 diagnostic tests. We encountered difficulty recruiting

	Skin Biopsy	AFT	GTT	Axon Flare
No. of Normal Control Subjects (target 40)				
41	✓	✓	✓	✓
No. of Osteoarthritis Controls (target 40)				
35	✓	✓	✓	✓
No. of SFPN subjects (target 40)				
46	✓	✓	✓	✓

Table 5. Status of subjects having undergone multiple tests under Task 2

osteoarthritis controls because, as a condition mostly of the elderly and/or overweight, they displayed a greater prevalence of impaired glucose tolerance (IGT) or other confounding conditions. We ultimately rejected 12 osteoarthritis subjects from the study for IGT or other pre-existing conditions.

Methods: Task 2d: We considered statistical significance, positive and negative predictive value, cost and practicality to determine which of the three tests, axon flare, skin biopsy, or AFT, were the best to carry forward in this study. Of the three, only axon flare measurement by laser Doppler flowmetry was judged by the American Academy of Neurology (AAN) to not have been adequately studied [1], so we compared this technique to AFT and skin biopsy which received evidence-based medicine Level B and Level C recommendations, respectively, by the AAN for diagnosis of small-fiber sensory neuropathy [1].

Outcomes: Task 2d: We first demonstrated that the skin neurite densities of normal and osteoarthritis controls, as judged by the number of participants with nerve fiber counts below the 5th centile of normal, were not significantly different, nor was the incidence of abnormal AFT results (see Table 6). For diagnostic purposes, it is generally accepted that values at or below the 5th centile are evidence of small-fiber polyneuropathy (abnormal), while those between the 5th and 15th centile are borderline, and those above the 15th centile are considered normal. Although the osteoarthritis control group was in general older than the normal control group, direct comparison of AFT and biopsy results are possible among the groups even though they are not age-matched because 1) the centiles we established for skin biopsy in Task 1 are age-corrected, and 2) interpretation of AFT results is performed with age-adjusted norms supplied the equipment manufacturer via data provided by the Mayo Clinic. We then determined that the incidence of abnormal AFT and skin biopsy results was higher among neuropathy patients than either of the control groups (see Table 7).

	Normal control group (n=41)	Osteoarthritis control group (n=35)	Fisher's exact test comparison
Age (yrs), mean [median] {range}	33 [30] {18-64}	56 [56] {23-85}	--
Abnormal AFT, no. [%]	1 [2.4%]	4 [11.4%]	p = 0.17
Abnormal skin biopsy, no. [%]	5 [12.2%]	5 [14.3%]	p = 1
Abnormal AFT and/or skin biopsy, no. [%]	5 [12.2%] *	8 [22.9%] *	p = 0.24

Table 6. AFT and skin biopsy results between the two control groups and comparison by Fisher's exact test. The incidence of abnormal test results was shown not to be statistically different between the groups (p>0.05).

* one subject in each group had both abnormal AFT and skin biopsy

	Combined control groups (n=76)	Neuropathy patients (n=46)	Fisher's exact test comparison
Age (yrs), mean [median] {range}	44 [44] {18-85}	48 [46] {20-80}	--
Abnormal AFT, no. [%]	5 [6.6%]	21 [45.6%]	$p < 0.001$
Abnormal skin biopsy, no. [%]	10 [13.2%]	16 [34.8%]	$p = 0.0063$
Abnormal AFT and/or skin biopsy, no. [%]	13 [17.1%] *	31 [67.4%] †	$p < 0.001$

Table 7. AFT and skin biopsy results between the combined control group and neuropathy patients, and comparison by Fisher's exact test. The incidence of abnormal test results was shown to be very statistically different between the groups ($p<0.05$).

* 2 subjects had both abnormal AFT and skin biopsy
† 6 subjects had both abnormal AFT and skin biopsy

The laser-Doppler flowmetry measurements yielded six measures of axon flare: Direct and indirect measurements of flare intensity induced by acetylcholine and by histamine, and direct and indirect measurement of flare size due to acetylcholine and to histamine. The predictive value of each flare measurement was calculated by constructing receiver operating characteristic (ROC) curves based on whether the measurements accurately identified a verified neuropathy patient versus a normal or osteoarthritis control subject. Figures 6 to 11 are the resulting ROC curves and areas under the curves (AUC). The analysis indicates that most measures have poor discriminatory power for SFPN, and only direct measurements of acetylcholine and histamine-induced flare intensity are "fair" tests (based on the ranges: AUC =1: Perfect test, $0.9<AUC<0.99$: Excellent test, $0.9<AUC<0.89$: Good test, $0.7<AUC<0.79$: Fair test, $0.51<AUC<0.69$: Poor test, AUC = 0.5: no discriminatory power).

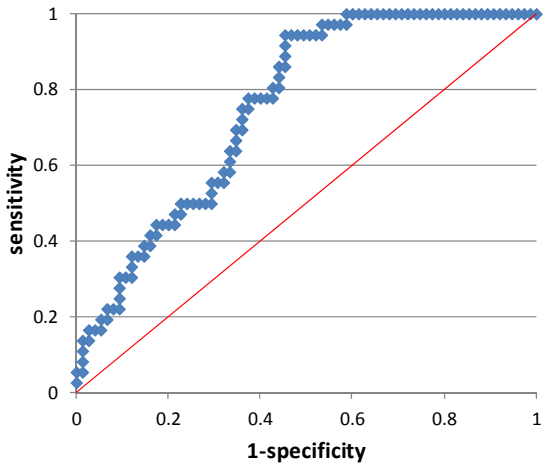

Figure 6. ROC curve of direct maximum flare due to acetylcholine. AUC = 0.76

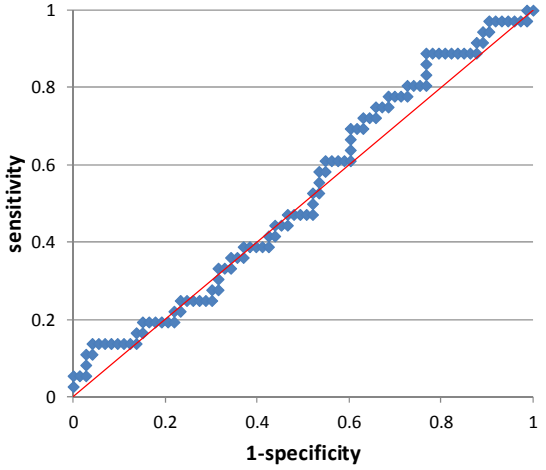

Figure 7. ROC curve of indirect maximum flare due to acteylcholine. AUC = 0.53

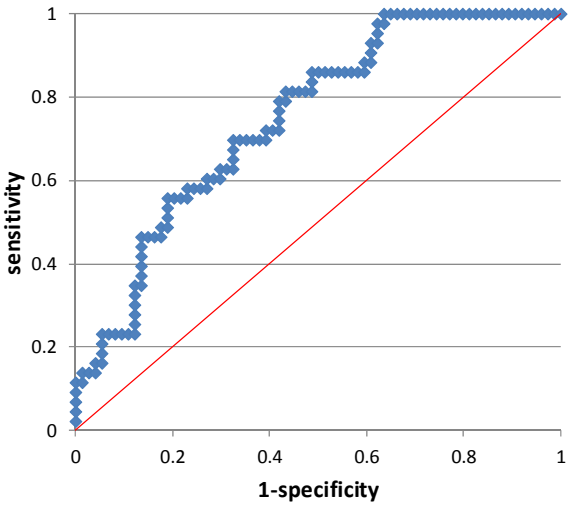

Figure 8. ROC curve of direct maximum flare intensity due to histamine. AUC = 0.75

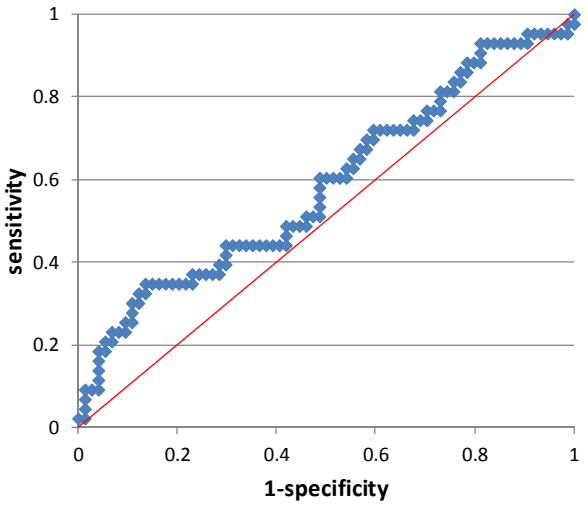

Figure 9. ROC curve of indirect maximum flare intensity due to histamine. AUC = 0.69

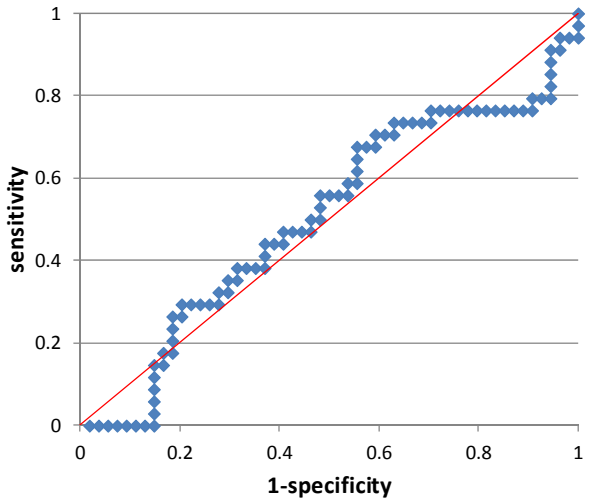

Figure 10. ROC curve of flare size due to acetylcholine. AUC = 0.50

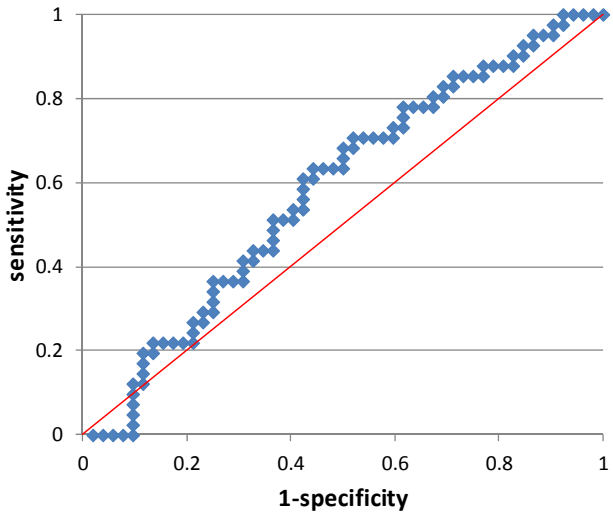

Figure 11. ROC curve of flare size due to histamine. AUC = 0.58

Summary of Outcomes for Task 2

We demonstrated in Task 1 that there is a strong age-related component to skin neurite density among normal controls. No age-related correlation or trend was found for axon flare response. Both direct acetylcholine and histamine flares were shown to be only fair tests for small-fiber polyneuropathy, otherwise diagnosed by 1) abnormal AFT, and/or 2) abnormal skin biopsy, and/or 3) neurological exam. Therefore we conclude that the best tests for polyneuropathy must include AFT, skin biopsy and neurological exam, and that axon flare is not sufficiently discriminatory. This is consistent with researchers who have done similar comparisons of diagnostic methods and posit that more than one test may be necessary [7]. Thus we applied the

three tests (AFT, skin biopsy, and neurological exam based on the Utah Early Neuropathy Scale (UENS, see Appendix 2)) to succeeding subjects in this study (Specific Aim II).

Specific Aim II. To use the best of these tests to determine the prevalence of SFPN among GW-ill veterans recruited with the assistance of the VA Decision Support System, and among subjects with fibromyalgia, and to compare SFPN prevalence to the prevalence in unaffected Gulf-War veterans and our demographically matched civilian controls.

Task 3: Determine prevalence of SFPN in Gulf War-ill veterans and in subjects diagnosed with fibromyalgia. The best tests identified above will be administered to groups of normal Gulf War veterans and veterans suffering from Gulf War Illness, and to subjects with fibromyalgia.

a. Recruit healthy and ill Gulf War veterans. Cohorts of 150 of each veteran group will be recruited by a combination of electronic medical-record searches at Mass. General Hospital, VA databases, and DoD databases. Additional IRB approvals external to MGH may be required. (months 18 – 30)

b. Test veteran cohorts with best test(s) of Task 3 to determine prevalence of SFPN among Gulf War Ill veterans. (months 22 – 30)

c. Recruit 30 subjects diagnosed with fibromyalgia and test them with the same tests as the veteran subjects to determine prevalence of SFPN among fibromyalgia subjects (note: this sub-task is funded in part by NIH grant K24NS059892). (months 24 - 28)

d. Data analysis to determine and compare the prevalence of SFPN in Gulf War ill and controls. Multivariate data analysis to determine which of the tests have greatest potential for clinical diagnostic use. Positive and negative predictive value, diagnostic sensitivity and specificity, invasiveness and cost will be considered. Tests that complement or overlap will be identified. (months 26 – 32)

e. To prepare and submit for publication a manuscript in a high-impact medical journal that will make these findings available world-side. (months 32 – 36)

Methods: Task 3a. We recruited Gulf War veterans using multiple strategies. We began recruiting veterans through in-house advertisement and through RSVP for Health. We posted IRB-approved flyers to further advertise this study and contacted other researchers working with Gulf War veterans to share strategies and to mutually promote our respective studies. We performed RPDR searches to identify veterans of the appropriate age to have served in the first Gulf War followed by free-text searches for mention of Gulf War service. We also contacted the Defense Manpower Data Center (DMDC) Data Request System (DRS) to conduct a search of military personnel who served in the first Gulf War and currently reside in the New England area (specifically Massachusetts, Rhode Island, New Hampshire, Maine, Vermont, Connecticut, and New York). We performed a manual cross-reference with Partners Healthcare's Longitudinal Medical Records (LMR) to identify the physicians of those Gulf War veterans who have been

examined or treated at an affiliated Partners Healthcare hospital. IRB regulations only allow us to contact veterans through their physicians, so we developed an IRB-approved letter of introduction that their physician could then forward about our study. We also engaged information technology assistance from the RPDR administrators to devise a semi-automated search that would cross-reference the DMDC dataset with RPDR records. We contacted approximately 100 Veterans Service Organizations to promote our study. We also worked with the National Service Officer and National Area Supervisor for Disabled American Veterans of Massachusetts who included our study brochure in mailings to Massachusetts veterans. We also approached area Veterans Health Administration (VHA) hospitals to advertise the study. Veterans were asked to produce their Service/Discharge record (DD 214) to verify their deployed service, and a VHA physician's letter attesting to a diagnosis of Gulf War Illness if they were to be included in the Gulf War-ill group.

Methods: Task 3b: The study methods for skin biopsy and autonomic function test are the same as described in Task 2. We added additional study instruments to help us identify similarities or differences among our veteran subjects and patients diagnosed with chronic multisymptom illnesses such as fibromyalgia. In addition to a brief neurological exam – the Utah Early Neuropathy Scale [12] – we also asked veterans to fill out several other validated questionnaires of neuropathic pain and quality of life (please refer to Table 8). We added a portion of VA Form 10-9009a to our veteran subjects questionnaires. This portion of VA Form 10-9009a asks subjects to describe their health concerns in 10 categories and is part of the evaluation by VHA physicians that is entered into the Gulf War Health Registry (please see Appendix 3). These examinations and questionnaires were intended to help us better understand the health of our groups of veterans and to establish an additional case history for them, and also to better understand the similarities among chronic multi-symptom illnesses, small-fiber polyneuropathy, and Gulf War Illness (see Task 3c).

Short Form (SF) 36 (Medical Outcomes Study) Health Survey: Physical and Mental Component Scores [8]
Michigan Neuropathy Screening Instrument (MNSI) Patient Version [9]
Short Form McGill Pain Questionnaire (SF-MPQ-2) [10]
Visual Analog Scale (VAS) for pain [11]
Excerpt from VA Form 10-9009a

Table 8. Questionnaires filled out by veterans

Methods: Task 3c: This subtask was added during the second quarter of Year 2. Text related to this addition is underlined in the Specific Aim II and Task 3 descriptions above (from the Statement of Work). The goal was to integrate a separate fibromyalgia study that was conducted concurrently in our laboratory with Specific Aim II of this study. This is consistent with VA acknowledgment that fibromyalgia may emerge during Gulf War service, but that veterans need not prove a connection between military service and their fibromyalgia in order to receive benefits; only that the fibromyalgia has to have emerged during active duty, and before December 31, 2016, and be at least 10 percent disabling. In addition, the VA has extended similar benefits to several “medically unexplained chronic multisymptom illnesses”. As a result, we surmised that although veterans are entered in the Gulf War Health Registry with multisymptom health complaints, few of those veterans currently seek a diagnosis of Gulf War Illness, and that fibromyalgia and other chronic multisymptom illnesses were emerging as a diagnosis preferentially to Gulf War Illness. The addition of this task allowed us to compare our

fibromylagia study results with each of our sets of veterans to more fully understand their chronic multisymptom illnesses, and to see if the prevalence of SFPN in veterans is similar that of fibromyalgia patients.

Outcomes: Task 3a Our initial recruitment strategies involving advertisement and contact with volunteers registered with RSVP for Health were not very successful. The DMDC search returned a dataset of 17,926 Gulf War veterans currently residing in the New England area; 3,192 in Massachusetts alone. When cross-matched with the Partners Healthcare System electronic medical records, we identified 730 veterans who were treated at Massachusetts General Hospital (MGH) or Brigham and Women's Hospital (BWH). Of those, the search found physicians for 286 veterans; 444 had no physician of record. Manual searches of the electronic medical records were done to identify them. Disabled American Veterans of Massachusetts identified 1,100 veterans in Massachusetts and we assisted with preparing a mailing that included our study brochure. Recruiting through local VHA hospitals was not successful because they asked that we first apply for permission through their individual IRBs, even to post our study brochures. Several Veterans Service Organizations were helpful, however.

Outcomes: Tasks 3b, 3d. In total we studied 38 Gulf War veterans, 3 with verified Gulf War illness (GWI) (one that was previously characterized as "Gulf War Ill" was reclassified as "Symptomatic" because of a lack of documentation) and 35 "controls". Among the controls, 12 were considered healthy and free of any GWI-type symptoms based on their screening information for entry into the study. However, as shown in Table 9, 23 (66%) of the "controls" had multi-symptom health complaints that they attributed to GWI, despite not having an official VHA diagnosis. We therefore distinguished between the "symptomatic controls" (symptomatic Gulf War veterans without a VHA diagnosis of Gulf War Illness) and the true "normal controls" (healthy Gulf War veterans with no symptoms, referred to as "non-symptomatic") based on the following series of preliminary results. Unless otherwise indicated, statistical differences were determined by two-tailed Student t-test with significance at $p < 0.05$.

Category	Studied
Gulf War Ill veterans	3
Healthy Gulf War veterans (non-symptomatic)	12
Symptomatic Gulf War veterans without diagnosis	23
Total	38

Table 9. Gulf War veterans tested with AFT and skin biopsy

A comparison of epidermal nerve fiber (ENF) density obtained by skin biopsy between Symptomatic and Non-symptomatic veterans (Figure 12) shows a cluster of lower ENF centiles with a lower median centile among Symptomatic veterans, but this was not a statistically significant difference for this

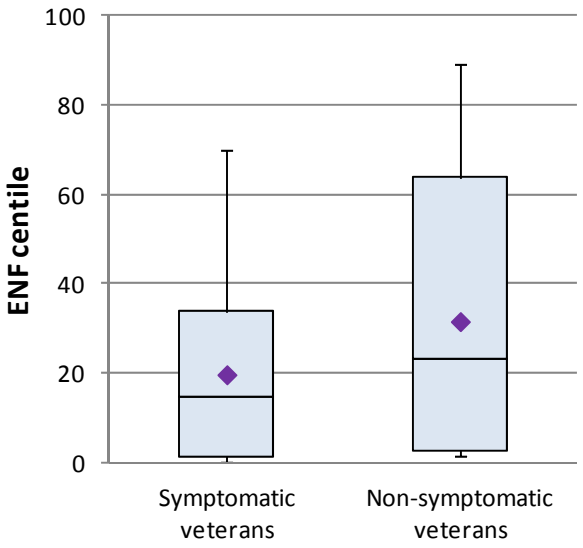

Figure 12. Comparison of ENF centiles of veterans without GWI diagnosis (symptomatic n=23, and healthy veterans n=12).

sample size (p=0.215). From the bottom, the boxes represent the 2nd and 3rd quartiles, the line between the boxes is the median value and the diamond is the mean. The top and bottom whiskers are the maximum and minimum centiles, respectively. The population centiles on which these analyses are based reflect the new norms established through Task 1. Figure 13, however, shows that there is a stronger trend toward a difference between all veterans (grouped together) and the normal control population (p=0.061), although the difference does not reach statistical significance.

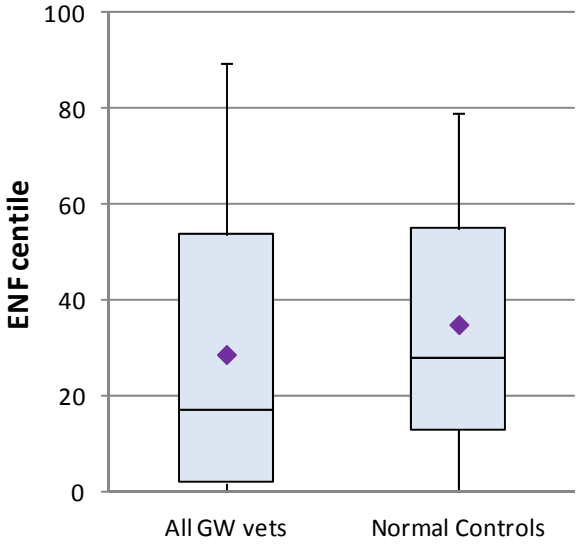

Figure 13. Comparison of ENF centiles of all veterans (n=38) and normal controls (n=41).

Presented below are neurite density data from the groups in this study who have undergone skin biopsy, autonomic function test, and glucose tolerance test (to rule out diabetic neuropathy). Figure 14 shows the centile of each biopsy gathered from those groups. The upper and lower dashed lines represent the cutoff for borderline (15th) and abnormal (5th) centiles, respectively. The black bars are the median centile for each group.

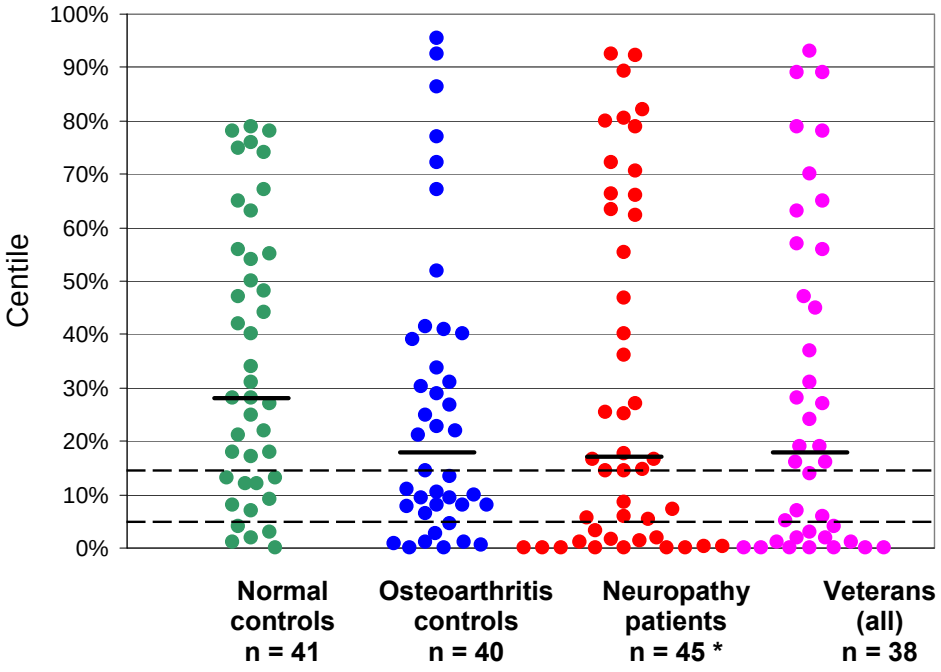

Figure 14. Centiles of each skin biopsy from the four study groups. * one neuropathy patient was biopsied by an outside lab and the results could not be assigned a centile

Figure 15 shows the distribution of normal, borderline, and abnormal skin biopsy centiles among the study groups. Qualitatively, from Figures 14 and 15, the veterans' neurite densities in aggregate more closely resemble the neuropathy patients than either of the control groups (normals and osteoarthritis), although the osteoarthritis group is closer in distribution to the veterans than the normal controls.

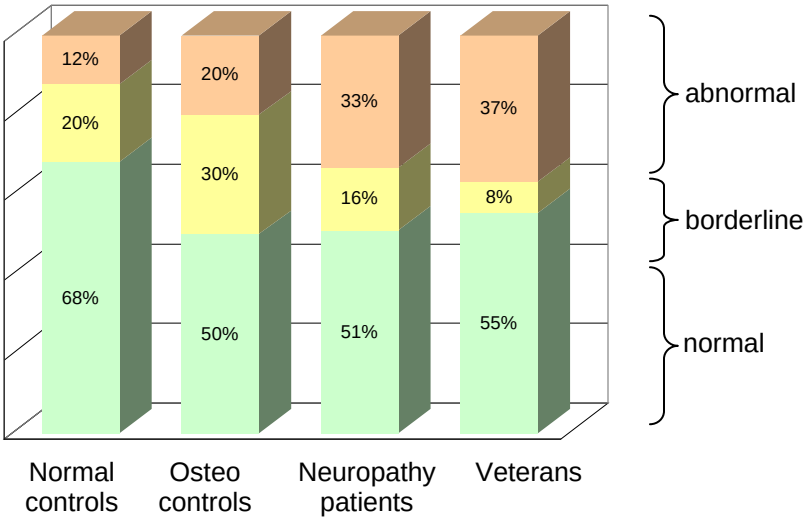

Figure 15. Distribution of skin biopsy centiles among the four study groups

Similar to the analysis that we performed in our publication on fibromyalgia [13] (see Task 3c) we looked at a dichotomous comparison of whether or not veterans have a significant number of objective markers of SFPN versus the control population for this study. Objective markers were defined as one or more abnormal test result from either skin biopsy or autonomic function test (AFT). Overall, 18 out of 38 veterans (47%) displayed one or more abnormal test results while only 13 out of 76 controls (17%) showed those markers of SFPN (Figure 16). The association between veteran status and abnormal test results indicative of SFPN was shown by Fisher's exact test to be very statistically significant ($p = 0.0014$) (Table 10).

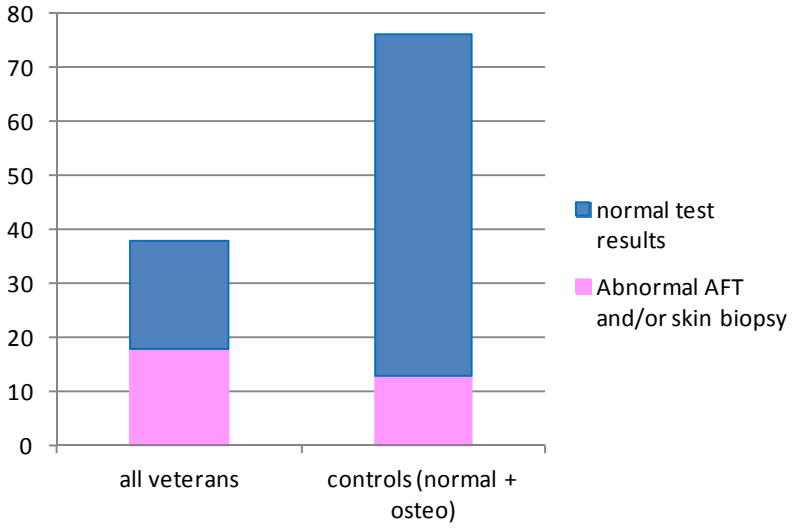

Figure 16. Comparison of the number of Gulf War veterans with objective markers of SFPN (abnormal test results) with all normal controls in this study.

	Combined control groups (n=76)	All veterans (n=38)	Fisher's exact test comparison
Age (yrs), mean [median] {range}	44 [44] {18-85}	50 [46] {40-65}	--
Abnormal AFT, no. [%]	5 [6.6%]	5 [13.2%]	p = 0.2971
Abnormal skin biopsy, no. [%]	10 [13.2%]	14 [36.8%]	p = 0.0064 **
Abnormal AFT and/or skin biopsy, no. [%]	13 [17.1%] *	18 [47.4%] †	p = 0.0014 **

Table 10. AFT and skin biopsy results between the combined control group and neuropathy patients, and comparison by Fisher's exact test. The incidence of abnormal test results was shown to be very statistically different between the groups ($p<0.05$).

* 2 subjects had both abnormal AFT and skin biopsy
† 1 veteran had both abnormal AFT and skin biopsy
** The association between veteran status and abnormal test results is considered to be very statistically significant.

To better understand any similarities among the multi-symptom complaints of the Symptomatic veterans, we scored and compared questionnaire responses among the Symptomatic, Non-symptomatic, and Gulf War ill veterans. Since we instituted these questionnaires, 12 symptomatic and 7 non-symptomatic veteran volunteers provided questionnaire responses for the MNSI, VAS, and SF-36 PCS and MCS. Fewer provided responses to the SF-MPQ-2. Also, 1 Gulf War-ill veteran provided complete questionnaire responses and 1 provided partial responses. The UENS exam was administered to 36 out of 38 veterans.

Figures 17 and 18 show veteran responses to the Michigan Neuropathy Screening Instrument (MNSI) and the Visual Analog Scale (VAS) for pain. The MNSI is designed to detect symptoms of neuropathy (based on signs of diabetic neuropathy). The range of possible scores is from 0 - 15, and a score > 2 is considered abnormal (indicative of neuropathy). The Visual Analog Scale measures pain severity on a scale of 0 ("no pain") to 11 ("pain as bad as it could be"). For the MNSI and VAS, statistically significant differences in responses between non-symptomatic and symptomatic veterans were observed. However, as with this and all the questionnaire scores to follow, there are too few Gulf War ill veterans to gauge statistically significant differences between their responses and the other groups'.

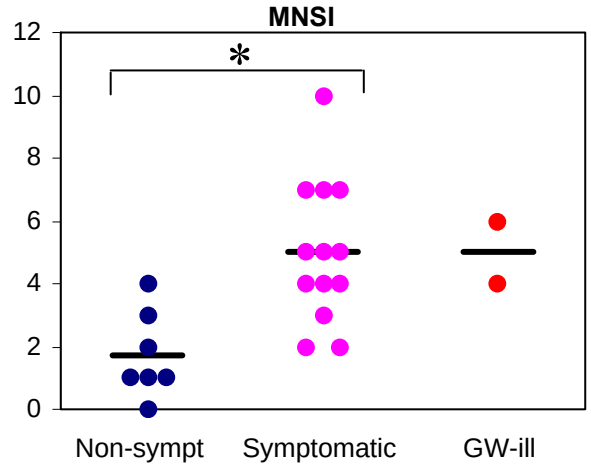

Figure 17. Scores from the Michigan Neuropathy Screening Instrument (MNSI) from Non-symptomatic, Symptomatic, and Gulf War ill veterans. Bars are mean scores. * denotes statistical significance (p = 0.0027).

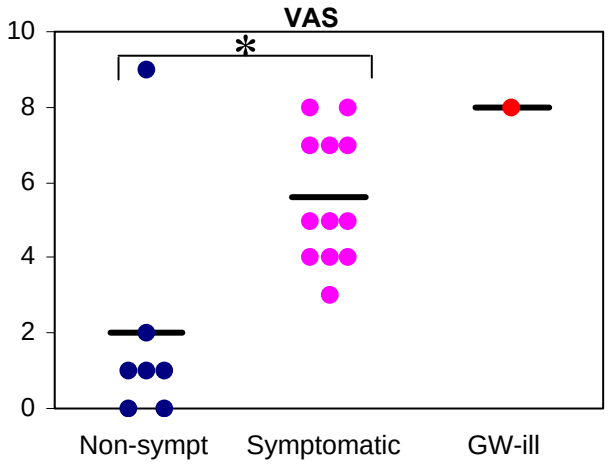

Figure 18. Scores from the Visual Analog Scale (VAS) for pain from Non-symptomatic, Symptomatic, and Gulf War ill veterans. Bars are mean scores. * denotes statistical significance (p = 0.0050).

Figure 19 shows veterans' scores from the Medical Outcomes Study Short Form-36 Physical and Mental Component Scores (SF-36 PCS and MCS). The scoring scales are normalized such that a score of 50 is considered normal, and scores lower than 50 indicate reduced physical or mental function/health. The responses indicate that symptomatic veterans were experiencing a more statistically significant reduction in physical health (PCS) than non-symptomatic veterans, but did not have a statistically different mental health outlook (MCS). The lack of a significant difference in MCS scores suggests that the veterans' multisymptom illness is physical and not psychogenic.

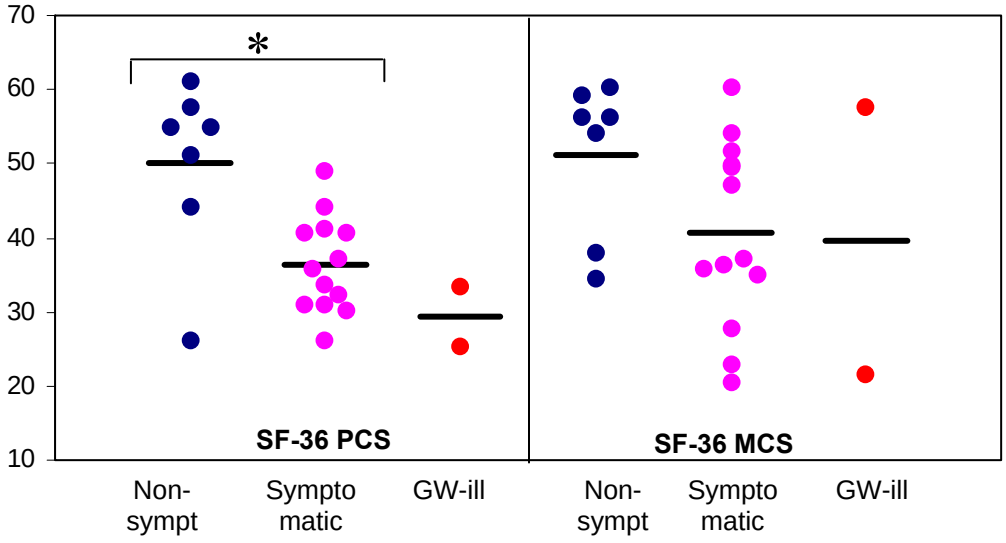

Figure 19. SF-36 Physical and Mental Component Scores (PCS and MCS) from Non-symptomatic, Symptomatic, and Gulf War ill veterans. Bars are mean scores. * denotes statistical significance (p = 0.0034).

Figure 20 shows scores from the revised Short Form McGill Pain Questionnaire (SF-MPQ-2) which is designed to assess the degree of continuous, intermittent, or neuropathic pain, or affective descriptors due to pain. Higher scores indicate more severe pain symptoms. Again, symptomatic veterans had significantly higher scores than non-symptomatic veterans.

Figure 21 shows the results of the brief neurological examination scored according to the Utah Early Neuropathy Scale (UENS). A higher UENS score indicates a greater degree of neuropathy. Although there is a trend toward increasing scores from non-symptomatic to symptomatic to Gulf War ill veterans, the differences are not statistically significant.

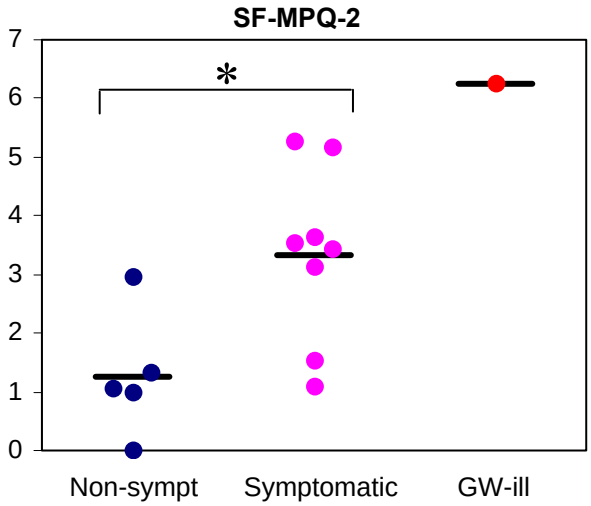

Figure 20. SF-MPQ-2 scores from Non-symptomatic, Symptomatic, and Gulf War ill veterans. Bars are mean scores.
* denotes statistical significance
(p = 0.0211).

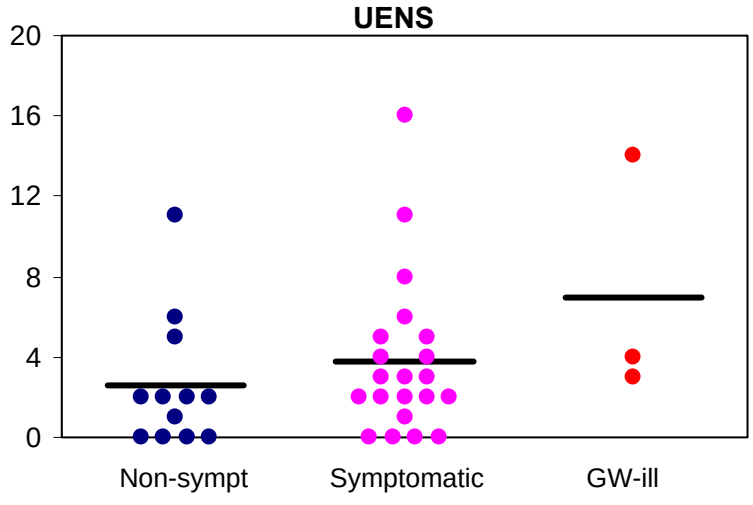

Figure 21. Scores of the UENS examination of Non-symptomatic, Symptomatic, and Gulf War ill veterans. Bars are mean scores. The scores were statistically not significantly different among the three groups.

Table 11 summarizes the comparison of questionnaire and UENS scores between symptomatic veterans and non-symptomatic veterans.

Test	Mean (symptomatic)	Mean (non-symptomatic)	p
MNSI	5	1.7	**0.0027 ***
VAS	5.6	2.0	**0.0050 ***
SF-36 PCS	36.4	50.1	**0.0034 ***
SF-36 MCS	40.6	51.2	0.0721
SF MPQ-2	3.3	1.3	**0.0211 ***
UENS	3.8	2.6	0.3879

Table 11. Questionnaires and UENS exam - comparing results among symptomatic versus non-symptomatic veterans

Outcomes: Task 3c. Our fibromyalgia study findings were published in 2013 in the journal PAIN® (the world's leading journal for pain research and anesthesiology) [13]. The manuscript is included as Appendix 4. In that study we looked for objective markers of small fiber polyneuropathy (SFPN) in patients previously diagnosed with fibromyalgia, and compared the results with those from a demographically matched set of normal controls. The results of that study mirror our findings among veterans. Among fibromyalgia patients we found that 50% had either abnormal skin biopsy or AFT, indicative of SFPN (compared with 47% of veterans in the current study) and only 17% of normal controls showed similar markers of SFPN (compared with 12% of normal controls in the current study).

Significantly, a large portion of the fibromyalgia patients with evidence of SFPN were found to have treatable causes of their SFPN. These causes were diagnosed through additional blood tests. This leaves open the possibility that veterans with evidence of SFPN may also have treatable causes of their neuropathy, which may be diagnosable if they underwent similar additional blood-testing. Also, it is significant to note that the most common blood-test abnormalities that we found among the fibromyalgia patients were linked to immune disorders that we previously associated with juvenile-onset SFPN in another study [14].

That study (included as Appendix 5) provided evidence of definite small-fiber polyneuropathy (SFPN) that developed at a young age in more than half of a group of patients. In 89% of the patients, blood abnormalities typical of immune disorders were found. Thus, when treated with immunomodulating therapies, most of those patients improved. This may be clinically significant for veterans in light of the findings of the objective markers of SFPN that were found in our veterans. Detailed histories and additional blood tests may reveal similar abnormalities that are as treatable as were the juvenile-onset patients.

The following results expand on the comparison between veteran groups, and the normal controls and fibromyalgia patients from the fibromyalgia study [13]. Presented here are additional analyses comparing MNSI scores (Figure 22), and SF 36 PCS and MCS scores (Figures 23 and 24) among the 3 categories of veterans and the fibromyalgia patients and controls whose results were already published [13]. Again, Symptomatic veterans (n=13) score similarly to Fibromyalgia patients (n=27) while Non-symptomatic veterans (n=7) score similarly to the normal control subjects (n=30) for each study instrument. There are still too few Gulf War-ill veterans (n=2) to make a valid comparison. All data points are means ± standard error from the mean (SEM).

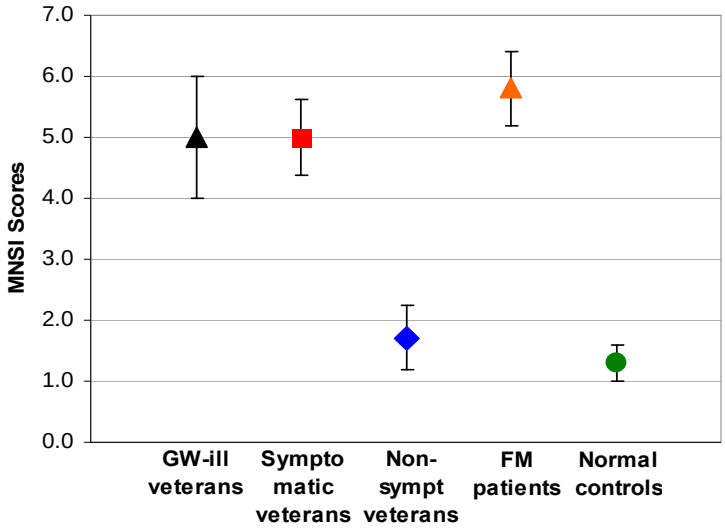

Figure 22. Comparison of MNSI scores among veterans, fibromyalgia (FM) patients and normal controls

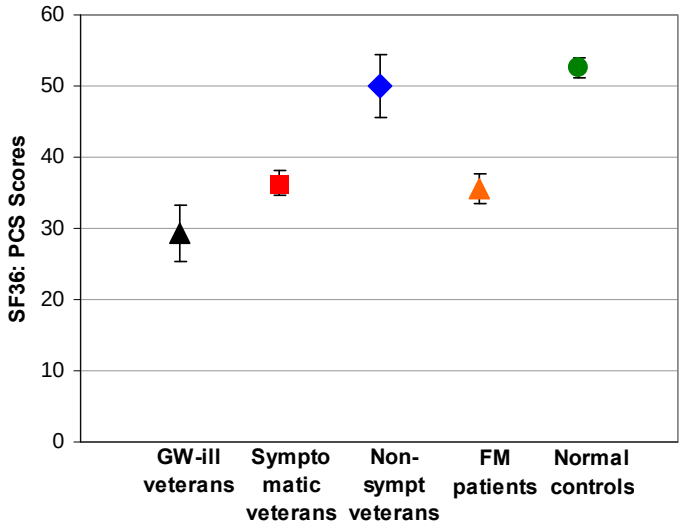

Figure 23. Comparison of SF 36 PCS scores among veterans, fibromyalgia (FM) patients and normal controls

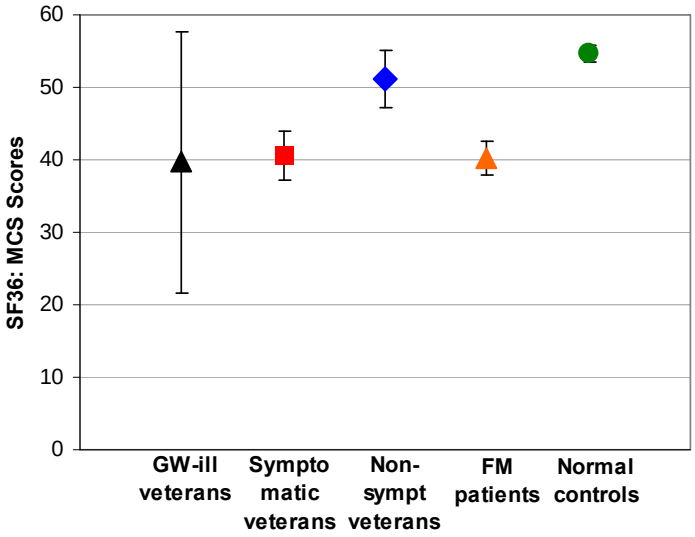

Figure 24. Comparison of SF 36 MCS scores among veterans, fibromyalgia (FM) patients and normal controls

We also compared the results of the Utah Early Neuropathy Scale (UENS) examination, which is a brief neurological exam intended to detect indications of neuropathy, between all veterans and the fibromyalgia patients and the matched controls from our fibromyalgia study. The comparison between fibromyalgia patients (FMS) and controls is already published [13]. Figure 25 adds the results from all veterans who underwent UENS testing. There is no statistical difference in all test results between our veterans (n=36) and the fibromyalgia patients (n=27), while both of those groups are statistically different from controls (n=26) in Motor, Pin, and Large Fiber tests.

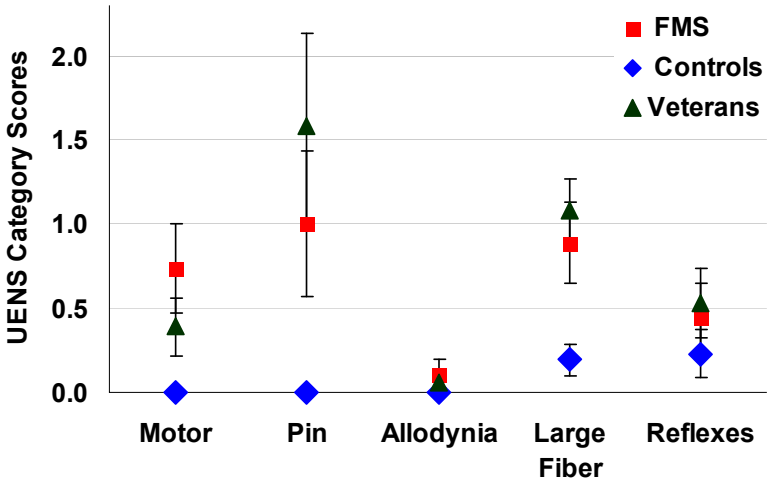

Figure 25. Comparison of Utah Early Neuropathy Scale scores for FMS patients, control subjects, and veterans by examination category: Motor (great toe extension); Pin (absent and reduced sensation); Allodynia (in toes and foot); Large Fiber Sensation (vibration and great toe position); and Reflexes (deep tendon at the ankle).

Summary of Outcomes for Task 3

We determined that Gulf War veterans in general had markers of SFPN in significantly greater proportion, based on abnormal autonomic function test and skin biopsy, than all the normal

controls. However, as we tested veterans, we found that there were also significant differences in results between healthy Gulf War veterans who were free of any health-related symptoms, and Gulf War veterans who had chronic multisymptom health complaints, but did not have an official diagnosis of Gulf War illness. We recognized that this symptomatic group had to be treated as a cohort separate from healthy veterans. We then compared these groups of veterans to a cohort of fibromylagia patients and controls with the same tests for SFPN, and also applied questionnaires that are validated for identifying neuropathy and its concurrent quality of life issues. We found that symptomatic veterans scored most similarly to fibromyalgia patients, and also found that veterans in general displayed markers of SFPN in approximately the same proportion as the fibromyalgia patients.

KEY RESEARCH ACCOMPLISHMENTS:

- Young subjects (below age 22) were found to show a "superabundance" of skin neurites. Although study of teenagers (below age 18) was not funded by this study, their neurite densities confirm the findings in the young adult subjects, and also anchored the lower end of the neurite density curve, thus providing a more accurate normative fit.
- Among normal controls, Asian subjects showed a significantly higher neurite density in the skin than non-Asian subjects
- Among normal controls, female subjects showed a statistically significant higher neurite density in the skin than male subjects
- There was insufficient power in the number of subjects in other ethnic groups to define significant differences in neurite densities
- We developed and now use for clinical diagnosis as well as for research (since 2012) a new normative neurite density scale for interpreting skin biopsies that provides normalized centiles taking into account age, gender, and ethnicity, based on a multivariate analysis of a logarithmic piece-wise linear regression model of the normative skin-biopsy data.
- This model was used to develop a neurite density calculator to provide more accurate norms and centiles for individual patients.
- A group of Gulf War veterans with multisymptom health complaints, but without any official diagnosis of Gulf War Illness or other diagnosis, was identified as significantly distinct from healthy veterans based on health questionnaires.
- Gulf War veterans in general were found to have statistically more objective markers of small-fiber polyneuropathy (based on autonomic function testing and skin biopsy) than normal controls.
- Half of fibromyalgia patients were found to have objective markers of polyneuropathy based on skin biopsy and autonomic function testing. Gulf War veterans had the same objective markers in approximately the same proportion.

REPORTABLE OUTCOMES:

Work that culminated in several abstracts prior to this study was carried over into this work to add significance to the findings. For instance, we presented preliminary results that indicated a

superabundance of skin neurites in youngsters and a dependence of skin neurite density on age, gender, and ethnicity [5]. This work was awarded a *Works in Progress* designation by the American Neurological Association (ANA) which identifies significant late-breaking research. We also retrospectively and prospectively explored which diagnostic tests may have better predictive value for small fiber polyneuropathy among a small initial cohort of SFPN patients and normal controls [15]. We since submitted an abstract on an extended normative biopsy series to the 139th meeting of the ANA, October 2014, which was accepted as a *Works in Progress*. We constructed an automated neurite density calculator based on the normative neurite density values that we collected, which we will make available to researchers and clinicians. A manuscript is in preparation to report these neurite density findings.

Our fibromyalgia findings were first presented at the 137th Annual Meeting of the ANA, Oct 2012. This work was also awarded a *Works in Progress* designation. These findings were subsequently published in the journal PAIN® [13]. A related study of juvenile-onset small-fiber polyneuropathy, to which support by the Gulf War Illness Research Program was also credited, was published in the prestigious journal Pediatrics [14]. Both these publications continue to garner attention and generate conversation on the role of small-fiber polyneuropathy in chronic multisymptom illnesses in general. The fibromyalgia study has been reported in newspapers nationwide, including USA Today (http://www.usatoday.com/story/news/nation/2013/12/15/fibromyalgia-research-breakthrough/3991063/), and a news segment on our juvenile-onset polyneuropathy study was aired on ABC news (http://abclocal.go.com/kabc/story?id=9246273).

CONCLUSION:

Through multivariate analysis, a new set of normative neurite densities obtained from skin biopsy provided a more accurate means of diagnosing small fiber polyneuropathy, taking into account the influence of age, gender, and ethnicity. We identified the need for skin biopsy and autonomic function testing, along with neurological exam, as the most useful objective tests for SFPN diagnosis; axon flare measurement by laser Doppler flowmetry was not as diagnostically accurate. We identified a new cohort of veterans (symptomatic but not officially diagnosed with Gulf War illness) that needed additional consideration and modification of our study design, to take into account the differences between their general health and that of healthy, non-symptomatic veterans.

Applying these tests we saw evidence of small fiber polyneuropathy (SFPN) in veteran subjects which parallels the findings of our high impact study of fibromyalgia patients. Since we identified treatable causes of SFPN in juvenile-onset cases, and found many of the same markers in fibromyalgia patients, the implication is that some veterans affected by similar chronic multisymptom illness (CMI) may have the same source of their illness, and thus have options for treatment. However, only additional blood tests, which were outside the scope of this study, can reveal if our veterans have the same treatable source of their chronic multisymptom illness or Gulf War Illness and thus have well-defined direction for their future care.

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Appendix 1

Statement of Work under W81XWH-10-1-0534

The Statement of Work was modified (at no additional cost) in October 2012 to include a separate study of fibromyalgia conducted in our laboratory, partly funded by Public Health Service (NINDS K24NS059892, UIL RR025758). The rationale was that there are many commonalities between Gulf War Illness (GWI) and fibromyalgia, so our FM results will likely raise interest in the community of GWI researchers and influence future research on GWI and other related chronic multisymptom illnesses (CMI).

The modifications to the original Statement of Work are underlined.

STATEMENT OF WORK

Undiagnosed small-fiber polyneuropathy - Is it a component of Gulf-War Illness?

Background: The term small-fiber polyneuropathy (SFPN) refers to body-wide dysfunction and degeneration of the small-diameter axons that transmit pain and control the body's autonomic (involuntary) functions. SFPN typically causes chronic pain, gastrointestinal symptoms, fatigue, dizziness, chronic headache, and skin abnormalities - complaints that overlap substantially with Gulf War Illness (GWI). Because SFPN produces vague, widespread symptoms, it is hard to diagnose clinically and requires special tests. In Aim I we will recruit, screen, and test normal control subjects and patients with definite SFPN from among the hundreds seen at Mass. General to compare the sensitivity and specificity of the best current tests (skin biopsy and comprehensive autonomic-function testing (AFT)), as well as a potential new test (axon flare reflexes to vasodilators). In Aim II, we will apply the best of these tests and compare results in Gulf War veterans with and without Gulf War illness to identify how often this diagnosable and treatable neurological illness is masquerading as GWI. We will apply the same tests to subjects diagnosed with fibromyalgia. By doing so, we will not only establish the relationship between Gulf War Illness and SFPN, and between SFPN and fibromyalgia, but will also determine which tests are the most diagnostically useful and should be re-engineered for more widespread clinical use. Most procedures in this study, including access to patient records, telephone screening, and skin biopsy and axon flare testing are already approved by the Partners Human Research Committee's Institutional Review Board (IRB) under protocol #1999-P-009042, "Laboratory Evaluation of Neuropathic Pain".

Specific Aim I. To determine which specific measurements of skin innervation, autonomic function, and skin blood flow provide the most sensitive, specific, and practical objective test for SFPN.

Task 1. Establish demographically correct skin biopsy norms. A cohort of 120 normal controls will be established to provide the necessary range of ages, sexes and ethnicities

a. Recruit, screen and test 120 normal controls. Some subjects have already been studied to provide preliminary data for this application. (months 1 – 6)

b. Multivariate data analysis to determine which of the three demographic variables tested (age, sex, race/ethnicity) influences the normal values for density of skin innervation and to generate the norms and limits between the normal and abnormal ranges necessary for clinical diagnostic use. (months 6 – 8)

c. To prepare and publish a manuscript in a high-impact neurological journal that will make these norms available for medical use world-side. An internet version will also be made available. (months 8 – 20)

Task 2. Compare the diagnostic sensitivity and specificity of skin biopsy, AFT, and axon-flare measurements to establish best tests for SFPN. Data will be collected from cohorts of 40 screened normal volunteers, SFPN patients, and symptom-matched control patients with severe osteoarthritis.

a. Recruit 40 normal subjects from among the 120 being studied by skin biopsy for Aim I for additional study with AFT and axon-flare measurements. (months 3 – 12)

b. Recruit 40 subjects with definite SFPN from among the several hundred already evaluated for clinical care at Mass. General Hospital by skin biopsy and AFT for additional study of axon-flare measurements. (months 8 – 18)

c. Recruit 40 severe osteoarthritis of the hip or knee from among the thousands such patients followed at Mass. General Hospital for study by skin biopsy, AFT, and axon-flare measurements. (months 8 – 18)

d. Multivariate data analysis to determine which of the tests have greatest potential for clinical diagnostic use. Positive and negative predictive value, diagnostic sensitivity and specificity, invasiveness and cost will be considered. Tests that complement or overlap will be identified. (months 18 – 22)

e. To prepare and publish a manuscript in a high-impact neurological journal that will make these recommendations available for medical use world-side. (months 22 – 34)

Specific Aim II. To use the best of these tests to determine the prevalence of SFPN among GW-ill veterans recruited with the assistance of the VA Decision Support System, and among subjects with fibromyalgia, and to compare SFPN prevalence to the prevalence in unaffected Gulf-War veterans and our demographically matched civilian controls.

Task 3: Determine prevalence of SFPN in Gulf War-ill veterans and in subjects diagnosed with fibromyalgia. The best tests identified above will be administered to groups of normal Gulf War veterans and veterans suffering from Gulf War Illness, and to subjects with fibromyalgia.

a. Recruit healthy and ill Gulf War veterans. Cohorts of 150 of each veteran group will be recruited by a combination of electronic medical-record searches at Mass. General Hospital, VA databases, and DoD databases. Additional IRB approvals external to MGH may be required. (months 18 – 30)

b. Test veteran cohorts with best test(s) of Task 3 to determine prevalence of SFPN among Gulf War Ill veterans. (months 22 – 30)

c. Recruit 30 subjects diagnosed with fibromyalgia and test them with the same tests as the veteran subjects to determine prevalence of SFPN among fibromyalgia subjects (note: this sub-task is funded in part by NIH grant K24NS059892). (months 24 - 28)

d. Data analysis to determine and compare the prevalence of SFPN in Gulf War ill and controls. Multivariate data analysis to determine which of the tests have greatest potential for clinical diagnostic use. Positive and negative predictive value, diagnostic sensitivity and specificity, invasiveness and cost will be considered. Tests that complement or overlap will be identified. (months 26 – 32)

e. To prepare and submit for publication a manuscript in a high-impact medical journal that will make these findings available world-side. (months 32 – 36)

Appendix 2. Utah Early Neuropathy Scale [12]

Patient Name:
Study Number:
Visit:
Date:

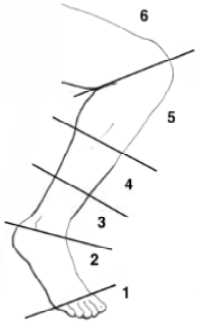
The Utah Early Neuropathy Scale

Motor Examination	Left	Right
0 normal		
2 weak		
Great Toe Extension		
Total Both Sides (out of 4)		

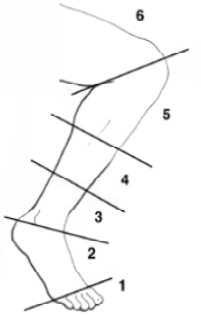
Pin Sensation:	L	R
0 normal		
1 for each segment with reduced sensation		
2 for each segment with absent sensation		
Total both sides (out of 24)		

Segments for pin sensation reporting

Left Leg



Right Leg



Allodynia/Hyperesthesia	L	R
0 normal		
1 if present in toes or foot		
Total both sides (out of 2)		

Mononeuropathy	L	R
CTS		
ULNAR		

Large Fiber Sensation	L	R
0 normal		
1 diminished		
2 absent		
Great toe vibration time	s	s
Great toe joint position		
Total both sides (out of 8)		

Deep Tendon Reflexe	L	R
0 normal		
1 diminished		
2 absent		
Ankle		
Total both sides (out of 4)		

Total Score (out of 42)

Appendix 3. Excerpt adapted from VA Form 10-9009a

Subject Initials:
Today's Date:
Subject ID:

10-9009A Form

1. Do you have gastrointestinal problems (diarrhea and/or abdominal pain)? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

2. Do you suffer from headaches and/or memory loss? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

3. Do you suffer from muscle aches and/or numbness? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

4. Have you been diagnosed with chronic fatigue? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

5. Do you suffer from joint pain? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

6. Do you have a chronic cough and/or shortness of breath? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

7. Do you have a skin rash? ☐ Yes ☐ No
If yes, please describe:_____
_____
_____

8. Do you suffer from vertigo (dizzy/light headed) and/or tinnitus(ringing in ears) ☐ Yes ☐ No
If yes, please describe: _____
_____
_____

9. Do you have any chest pain and/or palpitations? ☐ Yes ☐ No
If yes, please describe: _____
_____
_____

10. Do you have any reproductive concerns? ☐ Yes ☐ No
If yes, please describe: _____
_____
_____

Appendix 4

Fibromyalgia publication in PAIN®

This publication resulted from studies on fibromyalgia done in our laboratory. Although this work was a cost-neutral addition to the Statement of Work, the Gulf War Illness Research Program is credited with partial support of this study because several of the normal controls participated in both studies, and this publication has implications for future Gulf War Illness and other chronic multisymptom illness research.

PAIN® 154 (2013) 2310–2316

www.elsevier.com/locate/pain

Objective evidence that small-fiber polyneuropathy underlies some illnesses currently labeled as fibromyalgia

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 7 April 2013
Received in revised form 10 May 2013
Accepted 3 June 2013

Keywords:
Chronic pain
Human-subject research
Peripheral neuropathy
Peripheral nerve
Skin biopsy
Autonomic function testing

ABSTRACT

Fibromyalgia is a common, disabling syndrome that includes chronic widespread pain plus diverse additional symptoms. No specific objective abnormalities have been identified, which precludes definitive testing, disease-modifying treatments, and identification of causes. In contrast, small-fiber polyneuropathy (SFPN), despite causing similar symptoms, is definitionally a disease caused by the dysfunction and degeneration of peripheral small-fiber neurons. SFPN has established causes, some diagnosable and definitively treatable, eg, diabetes. To evaluate the hypothesis that some patients labeled as having fibromyalgia have unrecognized SFPN that is causing their illness symptoms, we analyzed SFPN-associated symptoms, neurological examinations, and pathological and physiological markers in 27 patients with fibromyalgia and in 30 matched normal controls. Patients with fibromyalgia had to satisfy the 2010 American College of Rheumatology criteria plus present evidence of a physician's actual diagnosis of fibromyalgia. The study's instruments comprised the Michigan Neuropathy Screening Instrument (MNSI), the Utah Early Neuropathy Scale (UENS), distal-leg neurodiagnostic skin biopsies, plus autonomic-function testing (AFT). We found that 41% of skin biopsies from subjects with fibromyalgia vs 3% of biopsies from control subjects were diagnostic for SFPN, and MNSI and UENS scores were higher in patients with fibromyalgia than in control subjects (all $P \leqslant 0.001$). Abnormal AFTs were equally prevalent, suggesting that fibromyalgia-associated SFPN is primarily somatic. Blood tests from subjects with fibromyalgia and SFPN-diagnostic skin biopsies provided insights into causes. All glucose tolerance tests were normal, but 8 subjects had dysimmune markers, 2 had hepatitis C serologies, and 1 family had apparent genetic causality. These findings suggest that some patients with chronic pain labeled as fibromyalgia have unrecognized SFPN, a distinct disease that can be tested for objectively and sometimes treated definitively.

1. Introduction

Fibromyalgia syndrome (FMS) is a collection of ill-defined symptoms that includes chronic widespread pain (CWP; defined as $\geqslant$3 months of axial, plus left- and right-side, plus upper- and lower-body pain [51]). FMS is common, having 1% to 5% prevalence in Western countries, affecting females 3 to 4 times more commonly than males, and conveying high health-care costs [29,49,50]. The American College of Rheumatology formulated and revised the diagnostic criteria that raised awareness of FMS and facilitated approval of medications to palliate symptoms [51,52], but the biological causes of FMS have remained unknown. Recent interpretations emphasize a biopsychosocial model in which "central sensitization" causes painful responses to stress and stimuli. However, the brain-imaging alterations in patients with FMS that engendered this hypothesis [17,22] were later reinterpreted as being nonspecific consequences rather than causal [21,40,47].

Small-fiber polyneuropathy (SFPN) is a neurological cause of CWP. Unlike FMS, SFPN has identifiable pathology, physiology and causes and thus is definitionally a disease. SFPN is caused by dysfunction and degeneration of the small-diameter unmyelinated (C-fibers) and thinly myelinated (A-delta) peripheral axons that mediate nociception. Symptoms usually begin distally with foot or leg pain, but advanced cases can spread proximally to involve the torso as well. Occasional patients begin with patchy, proximal or generalized (non–length-dependent) symptoms caused by attack directed at the neuronal cell bodies (ganglionopathy or neuronopathy) [16]. Many patients with SFPN also have

DOI of original article: http://dx.doi.org/10.1016/j.pain.2013.06.037
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0304-3959/$36.00
http://dx.doi.org/10.1016/j.pain.2013.06.001

cardiovascular, gastrointestinal, microvascular, or sweating complaints resulting from disturbed efferent effects of somatic and autonomic small-fibers on internal organs, blood vessels, and sweat glands [15,20].

SFPN often remains undiagnosed because complaints of CWP are subjective and nonspecific, and patients' strength and reflexes are usually preserved on examination. Diagnostic testing using surface electromyography and nerve-conduction studies is insensitive to SFPN. The best objective diagnostic tests for SFPN are distal-leg skin biopsy immunolabeled to reveal the density of small-fiber epidermal innervation (level C recommendation by the American Academy of Neurology; level A recommendation by the European Federation of Neurological Societies [8,28]), and autonomic function testing (AFT) of cardiovagal, adrenergic, and sudomotor small-fiber function (American Academy of Neurology level B recommendation [1,8]). Quantitative sensory testing is not recommended because it relies on subjective reports [12]. The best instruments for measuring the symptoms and signs of SFPN are those applied in this study. The value of diagnosing SFPN is that sometimes its causes can be identified and the disorder cured. Common causes of SFPN include diabetes, hematological malignancies, autoimmune conditions, infections, toxins (including medications), and genetic mutations [4,9].

SFPN and FMS have symptoms in common—not only multifocal CWP, but also Raynaud phenomena, dizziness, gastrointestinal and urological symptoms, fatigue, and headache [4], and many patients with SFPN report that their illness had been interpreted as FMS prior to diagnosis of SFPN. Electrodiagnostic testing of 58 patients labeled as having fibromyalgia for large-fiber demyelinating polyneuropathy found neuropathic abnormalities in 33% [6], and comparing sensory symptoms among 1434 patients with FMS and 1623 patients with painful diabetic polyneuropathy identified some commonalities [26]. Several recent abstracts suggest that SFPN and FMS may overlap [41,44,45]. Our discovery of objectively confirmed SFPN in 59% of patients with childhood-onset CWP [37], many originally labeled as having FMS, prompted this study.

2. Methods and subjects

2.1. Study design and subject recruitment

All procedures and protocols were approved by the institutional review board. Sample-size calculations assuming $\alpha = 0.05$ indicated that studying 33 subjects per group would have 90% power to detect a large effect size (0.8), and groups of 25 would have 80% power, so we planned for groups of 25 to 30. Recruitment strategies included print and e-mail advertising at Massachusetts General Hospital (MGH) and e-mail to MGH's Clinical Research database of $\geqslant$22,000 preregistered people interested in participation in clinical research (http://www.rsvpforhealth.partners.org/). No subjects were recruited from any neurology practice or clinic. We digitally searched MGH's medical records to identify patients with fibromyalgia, to whom we mailed a description of this study through their primary-care provider. We also recruited through fibromyalgia support groups on the social networking sites Facebook and Meetup.com. Respondents were telephone-screened for inclusion (see criteria below); medical records confirming prior FMS diagnoses were obtained; and all eligible respondents were invited for study (Fig. 1). Asymptomatic volunteers were selected on the basis of demographic characteristics that matched those of the cohort with FMS.

Inclusion criteria for all subjects were that they be at least 18 years old and have the ability to comprehend and execute the protocol. Exclusion criteria comprised inability to give informed consent and contraindication to AFT or skin biopsy. FMS subjects were additionally required to submit medical records that documented a prior independent physician's diagnosis of fibromyalgia, plus they were required to fulfill the American College of Rheumatology (ACR) 2010 diagnostic criteria for FMS as assessed by questionnaire [51]. These criteria require a score $\geqslant$7 on the widespread-pain inventory plus a score $\geqslant$5 on the symptom severity scale, or $\leqslant$3 on the widespread-pain inventory, or $\leqslant$6 if the symptom severity scale was $\geqslant$9 [51].

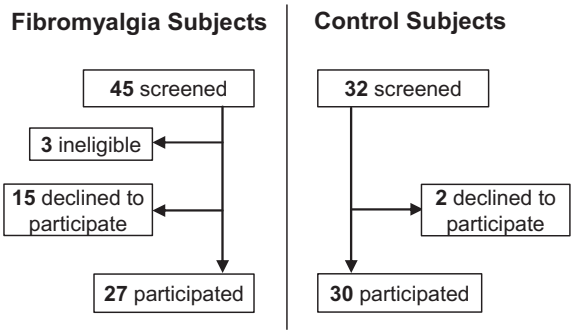

Fig. 1. Enrollment.

2.2. Data acquisition

To quantitate symptoms, subjects consented to complete the Michigan Neuropathy Screening Instrument questionnaire (MNSI), which consists of 15 yes/no questions concerning foot sensation, including pain, numbness and sensitivity to temperature [11,34]. For diabetic small-fiber–predominant polyneuropathy, the sensitivity of the MNSI was 80%, specificity was 95%, positive predictive value was 97%, and negative predictive value was 74% [11]. All subjects also underwent targeted neurological examination, which was codified using the Utah Early Neuropathy Scale (UENS), a neurological examination of the lower legs and feet designed to detect small-fiber–predominant sensory neuropathy [43]. It focuses particularly on loss of small-fiber–mediated pin-evoked nociception. To provide additional information about the representativeness of the FMS and control samples, all subjects completed the Beck Depression Inventory (BDI-21) and the Medical Outcomes Study Short Form Health Survey (SF-36). These validated questionnaires have known profiles among patients with FMS and normal controls [2,3,23]. Subjects were compensated $100 plus parking expenses. Data were managed using the Harvard Clinical and Translational Science Center's secure online Research Electronic Data Capture (REDCap) platform [18], and the accuracy of data entry was verified.

Neurodiagnostic skin biopsies were processed and analyzed by our accredited clinical diagnostic laboratory according to consensus standards [8,27]. We removed 2 or 3 mm diameter skin punches from the anesthetized standard distal-leg site. Free-floating 50 μm vertical sections were immunohistochemically labeled against PGP9.5, a pan-neuronal marker (Chemicon, Temecula, CA) to reveal epidermal nerve fibers (ENFs) and permit standard measurements of their density (Fig. 2) [4,8,28]. Almost all PGP9.5-immunoreactive epidermal neurites are nociceptive small-fiber endings, and axonal localization of epidermal PGP9.5 immunolabeling has been verified ultrastructurally [42,48]. A single skilled morphometrist blinded to group allocation measured ENF density. Our laboratory reports ENF densities per mm^2 skin surface area to control for varying skin-section thickness between laboratories. Skin-biopsy corroboration of SFPN required meeting the standard clinical diagnostic criteria for SFPN diagnosis, namely ENF density <5th centile of predicted laboratory norm [28]. Our laboratory's sex-, age-, and race-specific norms are based on the multivariate analysis of 240 screened biopsies from normal volunteers [25].

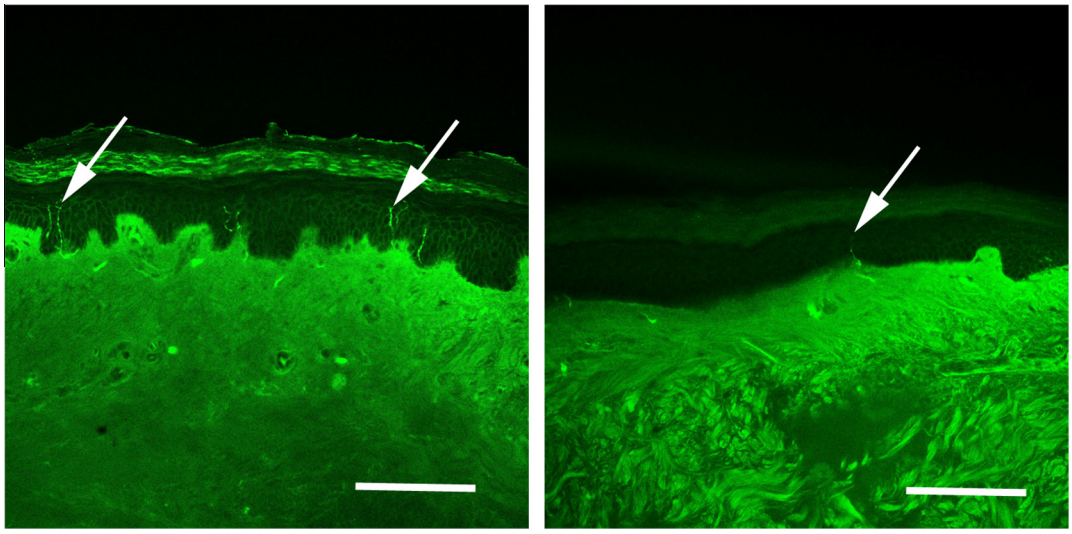

Fig. 2. Immunohistochemical visualization of sensory nerve endings in distal-leg skin biopsy by anti-PGP9.5-immunoreactivity. Arrows depict labeled axons. (A) Biopsy from 44-year-old Caucasian female, control subject with normal density of epidermal innervation (337 neurites/mm^2 skin surface area; at the 76th centile of predicted value). (B) Biopsy from 47-year-old Caucasian female, fibromyalgia subject with reduced density of epidermal innervation diagnostic for small-fiber polyneuropathy (135 neurites/mm^2 skin surface area; at the 3rd centile of predicted value). Bars represent 50 μm.

AFT was also performed using standard clinical diagnostic methods, equipment (WR Medical Electronics, Stillwater, MN), and interpretations recommended for SFPN diagnosis [1,8]. Subjects were directed to avoid wearing compressive clothing and to not smoke, eat or consume alcohol or caffeine for 4 h preceding testing. Their medications were reviewed, and potentially interfering medications were held for 24 to 48 h before testing (including all medications with anticholinergic effects, pain medications, antidepressants, antihistamines, cough and cold remedies, and cardiovascular modulators, including adrenergics, diuretics, antihypotensive agents, and attention-deficit medications [9,33]). We measured heart-rate variability to deep breathing (6/m while supine) and the Valsalva maneuver, hemodynamic responses to 80 degrees head-up tilt for 10 m, and acetylcholine-evoked sweat production. We applied the sole validated quantitative scoring system (Composite Autonomic Scoring Scale) [30] and later modifications [33,35,36], incorporating equipment-specific reference ranges. Corroboration of SFPN by AFT required at least 2 of 4 abnormal AFT subtest results. Standard sex- and age-adjusted definitions of abnormality were used: abnormal heart-rate response to deep breathing comprised <2.5th centile of predicted norm, and abnormal response to the Valsalva maneuver comprised no change in phase II, or Valsalva ratio <2.5th centile [33]. Abnormal tilt-table responses comprised change in heart rate $\geqslant$30, reductions in systolic blood-pressure $\geqslant$20 or reductions in diastolic blood-pressure $\geqslant$10 [13,33]. Abnormal sweat production comprised at least 2 sites with <50% of the 5th centile of norm for sex and age [33]. Because we used a Q-Sweat machine but published diagnostic norms use quantitative sudomotor axon reflex testing (QSART) units [32], we converted our measurements to QSART.

2.3. Outcome variables and data analysis

We followed consensus diagnostic criteria for SFPN in adults or children [7], and standard clinical practice in accepting any one or more objective-test results consistent with SFPN as confirming the diagnosis in a person who had sought medical help for otherwise unexplained symptoms consistent with SFPN [8,28]. Summary statistics comprised group means ± standard errors. Proportions of abnormal results were compared between groups by χ^2analysis.

3. Results

3.1. Validation of study groups

Table 1 demonstrates that the patients with fibromyalgia and control subjects were demographically similar, and that the patients with FMS were similar in age, sex and race to larger cohorts of patients with FMS, including those used to define the syndrome [39,51]. The mean age at onset of FMS in the current cohort was 28.8 ± 3.0 yr, and illness duration averaged 19.1 ± 2.7 yr. Scores on the BDI identified control subjects as not depressed and FMS subjects as mildly depressed on average, consistent with prior FMS cohorts [3,23]. The physical and mental component scores of the SF-36 identified control subjects as being at or near the population mean of 50, whereas patients with FMS had significantly lower scores, consistent with illness (both $P < 0.001$; Table 1). The mean mental component scores score in patients with FMS (40.2) was very similar to the mental component scores reported in larger FMS cohorts (38.6, 43.1), but their physical component scores (35.6) were less abnormal (28.0, 29.6), suggesting that the current sample may have had slightly less physical disability than historical FMS cohorts (as reviewed in [19]).

3.2. Symptoms and neurological-examination signs consistent with SFPN

The MNSI has a range of possible scores between 0 and 15, in which abnormal was previously defined as >2 [34]. The MNSI scores of 28 control subjects were, on average, in the normal range (1.3 ± 0.3), whereas scores of the 23 FMS subjects were, on average, abnormal and consistent with polyneuropathy (5.8 ± 0.6, $P \leqslant 0.001$; Table 1). Results of the UENS standardized neurological examination were available from 27 patients with FMS and 26 control subjects. UENS scores can range between 0 and 42, with the ideal score being 0. There is no established cutoff between normal and abnormal UENS scores, but the scores of patients with FMS were significantly higher than the scores of control subjects (3.1 ± 0.7 vs 0.5 ± 0.2, $P \leqslant 0.001$; Table 1), providing evidence that the FMS group but not the control group had examination findings consistent with SFPN. Fig. 3 depicts the differing categories of

Table 1
Summary statistics for all study participants.

Characteristic	Fibromyalgia subjects, n = 27	Control subjects, n = 30	*P* value
Demographic variables			
Age, mean yr (± SEM)	46.5 (2.3)	44.8 (1.9)	0.576
Age range, yr	26–68	25–65	–
Female sex, no.(%)	20 (74.1%)	24 (80.0%)	0.460
White race, no. (%)	21 (77.8%)	22 (73.3%)	0.361
Duration of fibromyalgia symptoms, mean yr (±SEM)	19.1 (2.7)		
Objective test results			
ENF density < 5%, no. (%)[a]	11 (40.7%)	1 (3.4%)	1.14×10^{-24}
Abnormal AFT, no. (%)[b]	5 (19.2%)	4 (13.3%)	0.271
One or more of the above, no. (%)[c]	13 (50.0%)	5 (17.2%)	2.40×10^{-5}
Signs and symptoms (range, healthy → ill)			
MNSI, mean ± SEM (range, 0–15; abnormal >2)	5.8 ± 0.6	1.3 ± 0.3	2.11×10^{-9}
UENS ± SEM (range, 0–42; ideal = 0)	3.1 ± 0.7	0.5 ± 0.2	6.21×10^{-4}
BDI, mean ± SEM (range, 0–63; ideal = 0)	14.4 ± 1.8	3.3 ± 0.8	4.35×10^{-7}
SF-36 PCS ± SEM (range, 100–0, μ = 50, SD = 10)	35.6 ± 2.1	52.5 ± 1.4	8.07×10^{-9}
SF-36 MCS ± SEM (range, 100–0, μ = 50, SD = 10)	40.2 ± 2.4	54.7 ± 1.2	1.26×10^{-6}

AFT, autonomic function test; BDI, Beck Depression Inventory; ENF, epidermal nerve fiber; MCS, mental component summary; MNSI, Michigan Neuropathy Screening Instrument; PCS, physical component summary; SEM, standard error of the mean; SF-36, Medical Outcomes Study Short Form Health Survey; SFPN, small-fiber polyneuropathy; UENS, Utah Early Neuropathy Scale.
[a] One control subject declined biopsy.
[b] One fibromyalgia subject withdrew from sweat test.
[c] Three FMS subjects had both abnormal ENF density and abnormal AFT results.

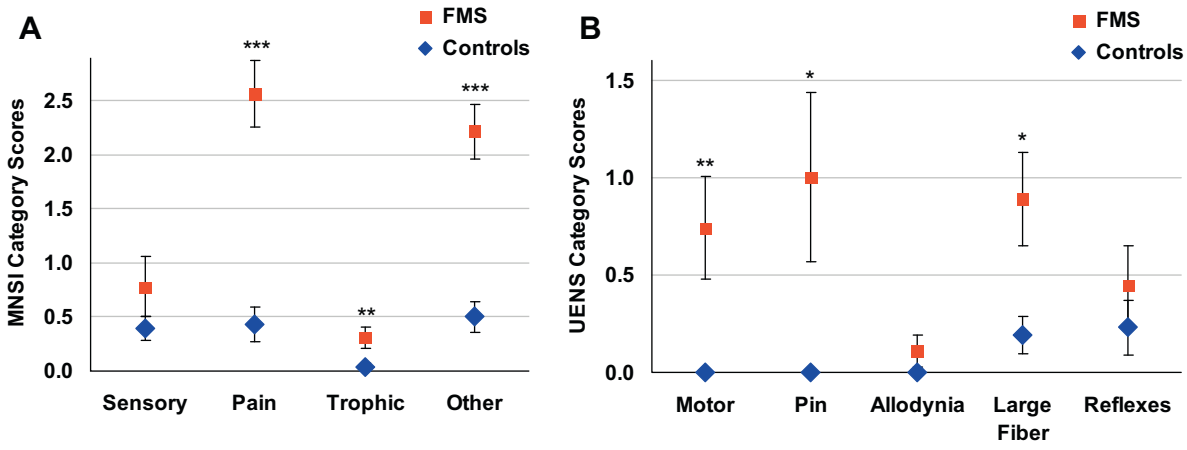

Fig. 3. Evidence of SFPN from neuropathy symptom scales. (A) Comparison of Michigan Neuropathy Screening Instrument scores for subjects with FMS and control subjects by questionnaire category. Sensory comprised MNSI questions 1, 7 and 13; pain comprised questions 2, 3, 5, 6, and 12; trophic comprised questions 8, 14 and 15; other comprised questions 4, 9, 10, and 11. (B) Comparison of Utah Early Neuropathy Scale scores for subjects with FMS and control subjects by examination category: motor (great-toe extension); pin (absent and reduced sensation); allodynia (in toes and foot); large fiber sensation (vibration and great-toe position); and reflexes (deep tendon at the ankle). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

results from the MNSI and UENS. The lack of allodynia among patients with FMS on both the UENS and the sensory category of the MNSI was of interest.

3.3. Objective tests corroborative of SFPN diagnosis

Overall, 50% of subjects with fibromyalgia as opposed to 17% of controls had one or more objective test results consistent with SFPN ($P \leqslant 0.001$; Table 1). Skin-biopsy results (Fig. 2) drove this difference, with 41% of biopsies from FMS patients vs 3% of biopsies from control subjects having ENF densities <5th centile of predicted laboratory norm ($P \leqslant 0.001$; Table 1). One control subject met skin-biopsy criteria for SFPN without having corresponding symptoms or signs (ENF <1st centile; MNSI score ⩽2; UENS score, 0). This was tentatively attributed to a concurrent diagnosis of multiple endocrine neoplasia-2, including thyroid cancer, which has been associated with axonopathy [5]. In contrast, AFT results were similar overall between patients with fibromyalgia and control subjects ($P = 0.27$; Table 1). One subject from each group had abnormally reduced heart-rate changes during deep breathing ($P = 0.46$); 7 FMS subjects vs 3 controls had abnormal responses to Valsalva ($P = 0.0006$); 9 subjects in each group had abnormal responses to tilt ($P = 0.50$); and 4 FMS subjects vs 0 controls had low sweat production at more than 1 site (P undefined).

3.4. Comparing results from fibromyalgia subjects with vs. without skin biopsies corroborative of SFPN

Given the highly significant differences in skin-biopsy results and the lack of differences in AFT results between FMS and control subjects, we performed a secondary analysis to compare the other test results between FMS subjects who did or did not meet skin-biopsy criteria for SFPN. Table 2 demonstrates that FMS subjects with SFPN-diagnostic skin biopsies were similar in sex and age

Table 2
Summary statistics comparing FMS subjects with or without objective evidence of SFPN.

Characteristic	FMS subjects with objective evidence of SFPN, n = 13	FMS subjects without objective evidence of SFPN, n = 14	*P* value
Demographic variables			
Age, mean yr (± SEM)	46.4 (2.8)	46.7 (3.7)	0.951
Age range, yr	26–63	28–68	–
Female sex, no. (%)	9 (69.2%)	11 (78.6%)[a]	0.404
White race, no. (%)	13 (100.0%)	8 (57.1%)	0.003
Outcomes (range, normal → abnormal)			
ENF, mean centile (± SEM)	3.8 (1.9)	48.2 (7.2)	0.00001
ENF density <5%, no. (%)[b]	11 (84.6%)	0 (0.0%)	
Abnormal AFT, no. (%)	5 (38.5%)	0 (0.0%)	
MNSI, mean ± SEM (range, 0–15; abnormal >2)	6.6 ± 0.6	4.7 ± 0.9	0.085
UENS ± SEM (range, 0–42; ideal, 0)	3.8 ± 1.2	2.5 ± 0.8	0.340
BDI, mean ± SEM (range, 0–63; ideal, 0)	18.5 ± 2.5	10.7 ± 2.2	0.028
SF-36 PCS ± SEM (range, 100–0, μ, 50, SD, 10)	35.5 ± 3.6	35.7 ± 2.3	0.963
SF-36 MCS ± SEM (range, 100–0, μ, 50, SD, 10)	35.1 ± 2.8	44.9 ± 3.4	0.038

AFT, autonomic function test; BDI, Beck Depression Inventory; ENF, epidermal nerve fiber; MCS, mental component summary; MNSI, Michigan Neuropathy Screening Instrument; PCS, physical component summary; SEM, standard error of the mean; SF-36, Medical Outcomes Study Short Form Health Survey; SFPN, small-fiber polyneuropathy; UENS, Utah Early Neuropathy Scale.
[a] 6 of 11 women were white.
[b] 3 FMS subjects had ENF density <5% plus abnormal AFT.

(70% female, mean age 46.4 ± 2.8 yr) to FMS subjects with nondiagnostic skin biopsies (77% female, mean age 46.7 ± 3.7 yr). However, all FMS/SFPN subjects were Caucasian, compared to only 57% of non-SFPN/FMS subjects ($P = 0.003$). The mean ENF centile value among FMS/SFPN subjects was 3.8 ± 1.9, whereas it was at the population mean (48.2 ± 7.2) among non-SFPN/FMS subjects ($P < 0.001$). Additionally, 39% of SFPN/FMS subjects had abnormal AFT results, whereas 0 non-SFPN/FMS subjects had such results. On the MNSI, UENS, BDI, and SF-36, both groups had largely similar results (Table 2).

3.5. Underlying causes of SFPN in patients with fibromyalgia

Testing for known causes of SFPN in FMS/SFPN subjects had not been planned, but it was requested by so many subjects with skin biopsies diagnostic for SFPN that we offered the recommended blood tests to all of them [9]. All 13 FMS/SFPN subjects had hemoglobin A_{1C} <6.0 mg/dL. For 2-h glucose-tolerance tests (75 g load) 8 of 11 subjects were normal according the current criteria of the American Diabetes Association. Two subjects had barely impaired fasting glucose levels (100 mg/dL and 102 mg/dL), one accompanied by marginal glucose intolerance (2 h = 140 mg/dL). The 2 FMS/SFPN subjects who did not undergo glucose-tolerance tests had normal random glucose levels (90 and 100 mg/dL). In contrast, 2 of 11 patients tested positive for hepatitis C, 1 a known case, the other a new diagnosis. All 13 subjects had noncontributory serum chemistries, blood counts, thyroid function, folate levels, triglycerides, C-reactive protein, angiotensin converting enzyme, tests for Lyme disease, lupus, Sjögren's disease, and celiac (IgA tissue-transglutaminase autoantibodies). None of the 12 subjects tested by immunofixation had monoclonal gammopathies. All of the 11 subjects tested had vitamin B_{12} levels within the reference range.

The most common blood-test abnormalities in subjects with FMS/SFPN were serological markers of dysimmunity that have been associated with juvenile-onset SFPN [37]. Specifically, 5 of 13 subjects had elevated erythrocyte sedimentation rates ($\geqslant$25 mm/h); 4 of 13 had antinuclear antibodies at titers greater than or equal to 1:160; and 3 of 13 had low complement C4, with 2 of 3 also having low complement C3 [37]. Overall, 62% of FMS/SFPN subjects had 1 or more of the markers of dysimmune function.

3.6. Adverse events

Skin biopsy caused no adverse events, but 1 subject suffered 2 small second-degree burns during sweat testing. Detailed investigation implicated faulty grounding electrodes, and there were no further events after new ground pads were substituted.

4. Discussion

The current results demonstrate that half of a small community-based sample of patients with FMS also had symptoms, signs, and objective test results that are accepted as diagnostic for SFPN, a biologically plausible cause of their symptoms of FMS. The difference between the labels FMS and SFPN is not merely semantic; SFPN is an established disease, and considerable information has been established about pathogenesis, whereas FMS is an aggregate of symptoms without prior evidence of a biological basis. Unlike FMS, SFPN can be objectively tested for (as here), and some causes of SFPN can be definitively treated, so advancing a patient's "diagnosis" beyond FMS to SFPN suggests potential causes, some of which can be tested for and treated. Skin biopsy (Fig. 2) corroborated SFPN in 41% of FMS subjects as opposed to 3% of normal controls, whereas both groups had similar results in autonomic function testing, implicating a primarily somatic and distal small-fiber polyneuropathy affecting pain neurons. This is consistent with the centrality of CWP rather than dysautonomic symptoms as the defining feature of the FMS phenotype.

This prospective study is limited by its small size. It was designed to detect large but not small effects, so AFT should be reevaluated in a larger cohort, particularly sweat production and responses to Valsalva for which FMS patients differed from controls. The observation that FMS subjects with SFPN were more likely to be Caucasians than those without SFPN ($P = 0.003$) also needs reevaluation in a larger study. However small, our FMS group was demographically representative of larger FMS cohorts, eg, the 258 patients from whom the current ACR diagnostic criteria were derived (92% female, mean age 55 yr, 87% non-Hispanic white [51]) and a community-based cohort of 10,129 patients with FMS (63% female, mean age 46 yr, races not reported [39]). Another limitation is the uncertain sensitivity and specificity of skin biopsy and autonomic function testing. There is no absolute or gold-standard

diagnostic test for SFPN against which to measure them, so their sensitivity and specificity cannot be defined except as relative to each other. Even these best-available tests are imperfect; indeed 13% of control subjects had abnormal AFT results. Tilt-table abnormalities were most common, consistent with the prevalence of orthostatic hypotension in the general population [31]. In contrast, skin biopsy, which measures late-stage axonal degeneration, had 97% specificity among control subjects in the current study. Another limitation is that we did not test subjects for other types of neuropathy, such as demyelinating neuropathies, motor or large-fiber sensory axonopathies, focal lesions, and auditory neuropathies. There is increasing recognition that different types of neurons are affected to varying extents in most polyneuropathies, regardless of how they are classified, particularly in severe or long-standing cases, and evidence of large-fiber motor or sensory polyneuropathy does not invalidate the presence of small-fiber polyneuropathy.

Strengths of the study included recruitment from the community so as to minimize referral or investigator bias, and use of rigorous inclusion criteria for FMS. We required both a preexisting, independent FMS diagnosis plus satisfying ACR research criteria, because they can be discordant [24]. The similarly rigorous and consensus-based evaluation for SFPN was an additional strength. Of note, both physiological (AFT) and anatomical tests for small-fiber polyneuropathy were applied. We also included assessments of depression and disability, which helped to validate the representative nature of the study samples.

Another strength is that we acquired data regarding potential underlying causes of SFPN in subjects with SFPN-diagnostic skin biopsies. We did not study controls because the definition of *abnormal* in clinical blood tests is based on extensive testing in normal as well as non-normal samples. Although diabetes is the most common cause of SFPN in developed countries, we found no evidence of causal contributions from diabetes or hyperglycemia. In contrast, 2 of 11 patients tested positive for hepatitis C, including 1 previously undiagnosed patient who was referred for treatment of hepatitis C because most cases are now curable. Among our patients, several had serological markers of autoimmunity that have been associated with juvenile-onset SFPN, a newly characterized type of SFPN that is associated with organ-specific, complement-consuming, humoral dysimmunity and with some favorable responses to immunomodulatory treatments [37,38]. Although most of the FMS subjects were middle-aged at the time of study, they reported onset of their FMS in youth, meaning that some could have had long-standing cases of juvenile-onset SFPN.

It was found that 3 of the subjects with FM/SFPN were related—a mother and 2 daughters. Given these study results, other members of their family who also suffered from juvenile-onset FMS sought evaluation for SFPN, leading to revised diagnoses of objectively confirmed SFPN in at least 6 family members in 4 generations. A genetic cause was presumed, and genetic evaluation was recommended. The well-known hereditary sensory and autonomic neuropathies were considered unlikely because of the absence of trophic signs (eg, painless ulcers). SFPN has been linked more recently to gain-of-function mutations in the *SCN9A* gene encoding the Nav1.7 sodium-channel isoform that is preferentially expressed in small-fiber nociceptive neurons, but commercial tests are not yet widely available [10]. FMS has also been associated recently with a specific *SCN9A* polymorphism [46], providing potential additional evidence of the convergence between FMS and SFPN. Although there is no cure for the genetic forms of SFPN yet, a trial of L-serine is under way for hereditary sensory and autonomic neuropathy-1 [14], and identifying specific mutations can guide patients toward targeted therapies not usually considered for patients with FMS, such as sodium-channel blockers.

In conclusion, this study provides objective evidence that almost half of a small sample of patients labeled with FMS have objective evidence of a neurological cause of their CWP and other symptoms of fibromyalgia, namely SFPN, a distinct peripheral-nerve disease. Blood tests in the small group of SFPN-diagnosed patients suggested that known causes of SFPN in the young (dysimmune, genetic, and infectious) were more common than causes of SFPN in maturity (eg, diabetes, cancer, vitamin deficiencies, or toxins), and they identified some treatable or curable causes such as hepatitis C. Patients currently carrying the FMS label may wish to discuss with their physicians whether testing for SFPN and its underlying causes might help them.

Conflicts of interest statement

None of the authors have conflicts of interest with respect to this work.

Acknowledgements

Supported in part by the Public Health Service (NINDS K24NS059892, UIL RR025758), the Department of Defense Gulf War Illness Research Program (GW093049), and a charitable donation from Ms. Jane Cheever Powell. Presented in abstract form to the 2012 meeting of the American Neurological Association. We thank the subjects for their participation and Kate O'Neil and Siena Napoleon for their assistance.

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Appendix 5

Publication on juvenile-onset small fiber polyneuropathy in Pediatrics

This publication resulted from studies on small-fiber polyneuropathy done in our laboratory. The Gulf War Illness Research Program is credited with partial support of this study because several of the adult normal controls participated in both studies, and this publication points to a potential source of the small-fiber polyneuropathy being identified in Gulf War veterans.

Evidence of Small-Fiber Polyneuropathy in Unexplained, Juvenile-Onset, Widespread Pain Syndromes

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KEY WORDS

peripheral nervous system disease, widespread chronic pain, dysautonomia

ABBREVIATIONS

AFT—autonomic function testing
CIDP—chronic inflammatory demyelinating polyneuropathy
CWP—chronic widespread pain
ENF—epidermal nerve fiber
ESR—erythrocyte sedimentation rate
GBS—Guillain-Barré syndrome, (acute inflammatory demyelinating polyneuropathy)
IVIG—intravenous immune globulin
POTS—postural orthostasis tachycardia syndrome
RR—reference range (of normal values for laboratory tests)
SFPN—small-fiber polyneuropathy

Dr Oaklander conceptualized and designed the study, obtained funding, extracted the data, participated in the data analysis, and drafted the initial manuscript and the rewrites. Dr Klein performed the autonomic function testing on the normal control subjects, participated in the data analysis, contributed to drafting and editing the figures, contributed to rewriting the manuscript, and approved the submitted and all revised versions of the manuscript.

This work was presented in abstract form to the American Neurologic Association (September 25–27, 2011; Manchester Grand Hyatt, San Diego, CA) and the Peripheral Nerve Society (June 25–29, 2011; Bolger Center, Potomac, MD).

www.pediatrics.org/cgi/doi/10.1542/peds.2012-2597

doi:10.1542/peds.2012-2597

Accepted for publication Dec 12, 2012

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PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

FINANCIAL DISCLOSURE: *The authors have indicated they have no financial relationships relevant to this article to disclose.*

FUNDING: Supported in part by the Public Health Service (K24NS059892), the Department of Defense (GW093049), and the Bradley and Curvey Family Foundations.

WHAT'S KNOWN ON THIS SUBJECT: Acquired widespread pain syndromes of youth are prevalent, disabling, usually unexplained, and untreatable. Small-fiber polyneuropathy causes widespread pain and multisystem complaints in older adults. Some causes are treatable. Neurodiagnostic skin biopsy, autonomic function testing, and nerve biopsy permit objective diagnosis.

WHAT THIS STUDY ADDS: It identifies definite (in 59%) and probable (in 17%) small-fiber polyneuropathy among 41 young patients with otherwise-unexplained, childhood-onset widespread pain. It characterizes this new disease's clinical features, diagnostic, and treatment options. Some cases appeared immune mediated and responded to immunomodulatory therapies.

abstract

OBJECTIVE: We tested the hypothesis that acquired small-fiber polyneuropathy (SFPN), previously uncharacterized in children, contributes to unexplained pediatric widespread pain syndromes.

METHODS: Forty-one consecutive patients evaluated for unexplained widespread pain beginning before age 21 had medical records comprehensively analyzed regarding objective diagnostic testing for SFPN (neurodiagnostic skin biopsy, nerve biopsy, and autonomic function testing), plus histories, symptoms, signs, other tests, and treatments. Healthy, demographically matched volunteers provided normal controls for SFPN tests.

RESULTS: Age at illness onset averaged 12.3 ± 5.7 years; 73% among this poly-ethnic sample were female (P = .001). Sixty-eight percent were chronically disabled, and 68% had hospitalizations. Objective testing diagnosed definite SFPN in 59%, probable SFPN in 17%, and possible SFPN in 22%. Only 1 of 41 had entirely normal SFPN test results. Ninety-eight percent of patients had other somatic complaints consistent with SFPN dysautonomia (90% cardiovascular, 82% gastrointestinal, and 34% urologic), 83% reported chronic fatigue, and 63% had chronic headache. Neurologic examinations identified reduced sensation in 68% and vasomotor abnormalities in 55%, including 23% with erythromelalgia. Exhaustive investigations for SFPN causality identified only history of autoimmune illnesses in 33% and serologic markers of disordered immunity in 89%. Treatment with corticosteroids and/or intravenous immune globulin objectively and subjectively benefited 80% of patients (12/15).

CONCLUSIONS: More than half among a large series of patients with childhood-onset, unexplained chronic widespread pain met rigorous, multitest, diagnostic criteria for SFPN, which extends the age range of acquired SFPN into early childhood. Some cases appeared immune-mediated and improved with immunomodulatory therapies. *Pediatrics* 2013;131:e1091–e1100

Syndromes involving unexplained chronic widespread pain (CWP) are prevalent and problematic in children and adults. The American College of Rheumatology defines CWP as pain affecting the axial, plus upper and lower body, plus the left- and right-side lasting ≥3 months.[1] Uncertainty about the etiology and pathogenesis of CWP precludes effective treatment with disease-modifying therapies, and chronic administration of pain-palliating medications has medical and social adverse effects that are even less acceptable in children than in adults. These increasingly recognized juvenile CWP syndromes often disrupt entire families and interfere with children's education and development.[2–4] CWP syndromes often include other unexplained complaints including dizziness, fatigue, headache, and nausea. More than half of juvenile patients with dizziness attributed to postural orthostasis tachycardia syndrome (POTS) also had chronic headache, abdominal pain, and/or fatigue,[5] suggesting a possible common etiology.

Small-fiber predominant polyneuropathy (SFPN) is a plausible biological substrate for CWP. SFPN refers to widespread damage predominantly affecting the small-diameter unmyelinated (C-fibers) or thinly myelinated (A-δ) peripheral axons that protect organisms by signaling pain upon harmful contact. SFPN usually begins in the longest axons, causing distal-onset or distal-predominant pain (in the feet). Occasional patients develop patchy or predominantly proximal symptoms attributed to proximal attack on the neuronal cell bodies (ganglionopathy/neuronopathy; Fig 2A).[6] Because small fibers also densely innervate and regulate the tone of microvessels, abnormal appearance (eg, edema, color changes) is common in affected areas.[7] Small-fibers were formerly dichotomized as somatic versus autonomic, a distinction blurred by recent discoveries that somatic/nociceptive axons have efferent and trophic effects formerly considered autonomic,[8] and that axons innervating sweat glands and blood vessels express the $TRPV_1$/capsaicin pain receptor.[9] Diagnosing SFPN is difficult because familiar signs of large-fiber neuropathy are absent or minimal, and electrodiagnostic testing is insensitive. The tests recommended for objective SFPN diagnosis were applied here: specifically distal-leg skin biopsy immunolabeled to reveal nociceptive epidermal nerve-fibers (ENF)[10] (level C recommendation by the American Academy of Neurology, level A recommendation by the European Federation of Neurologic Societies),[11,12] and autonomic function testing (AFT). This consists of 4 validated tests of cardiovagal, adrenergic, and sudomotor small-fiber function (level B recommendation by the American Academy of Neurology).[12,13]

Polyneuropathy has been believed to be rare in children, consisting of occasional cases of acute Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) caused by immune attack on large myelinated motor axons.[14] SFPN, in which small-diameter C- and A-δ fibers are preferentially damaged, was little known in children except for vanishingly rare genetic cases.[14,15] Isolated cases of erythromelalgia (aka erythermalgia), a historic phenotype comprising burning pain, redness, edema, and relief from cooling,[16] have been described in children[17] and some cases with prepubertal onset have been linked to sodium channel mutations.[18,19] Skin biopsy was the key to diagnosing SFPN in a Japanese 12 year old with new widespread pain and gastroparesis.[20] We and others have reported cases of teenagers with acute erythromelalgia and dysautonomia where skin biopsy confirmed severe SFPN, and corticosteroid treatment was curative.[21,22] Similar steroid-responsive cases were then described in younger children.[23,24] Children represent 4 of 21 cases of a recently characterized, acute small- plus large-fiber polyneuropathy,[25] and recent studies link pediatric erythromelalgia in children to SFPN, just as in adults.[26,27] Several new preliminary reports provide objective evidence that SFPN is prevalent in adult fibromyalgia, including our study identifying SFPN in 50% of fibromyalgia patients versus 0% of matched controls.[28–31]

METHODS

Selection of Cases and Records

After Institutional Review Board approval, outpatient records were screened to select subjects. Inclusion criteria were medical care by author A.L.O. between April 2007 and April 2011 for widespread multifocal pain (present in >1 limb or body region) beginning before age 21. Exclusion criteria were an identified objective cause of the pain. We obtained and read all available records extracting all provider notes, laboratory, physiology, pathology, and radiology reports. All technically adequate test results from academic or commercial laboratories were included and interpreted as reported or by using age-normed standard reference ranges.[32]

Primary Outcome: Objective Diagnosis of SFPN

There are no consensus diagnostic criteria for SFPN in adults or children,[33] so we integrated the results of all recommended objective diagnostic tests; PGP9.5-immunolabeled, distal-leg skin biopsy, AFT, and sensory nerve biopsy.[11,12] Confirmed SFPN required ≥1 definite objective-test SFPN diagnosis. Probable SFPN required minor abnormalities on ≥2 different tests, and possible SFPN required ≥1 minor objective abnormality. Electrodiagnostic testing (electromyography and nerve

conduction testing) was excluded because these tests do not capture small-fiber function, and quantitative sensory testing was excluded because it is a subjective test based on subject report.[34] A definitively abnormal skin biopsy required diagnosis of SFPN in the report or meeting standard diagnostic criteria (density of epidermal nerve fibers [ENFs] ≤ fifth centile of laboratory norms). Minor skin biopsy abnormalities comprised borderline ENF densities (5.1–15th centile or report of excess axon swellings).[35] A definitively abnormal AFT required SFPN diagnosis in the report or meeting standard diagnostic criteria (definite abnormalities in >1 domain). Minor AFT abnormalities required report of borderline, mild, minimal, or isolated abnormalities. A definitively abnormal nerve biopsy required SFPN diagnosis based on ultrastructural visualization of loss of unmyelinated axons.

Skin biopsy testing for SFPN has been standardized.[12,36] One 2- to 3-mm-diameter skin biopsy punch is removed from an anesthetized site on the distal leg and then vertically sectioned and immunolabeled against PGP9.5, a pan-neuronal marker, to visualize ENF and permit measuring axon density (Fig 1).[12,37] We report ENF densities per square millimeter of skin surface area to control for different laboratories' varying skin-section thickness. Most skin biopsies were interpreted at the Massachusetts General Hospital, whose norms come from biopsying 240 screened normal volunteers 14 to 86 years old.[38] Biopsies from patients <14 years old (n = 6) were normed to age 14 because of lack of norms for younger children. Performance of skin biopsies caused no adverse events.

AFT, considered more sensitive than skin biopsy,[39] has previously detected autonomic SFPN in half of adults with POTS.[40] Because AFT norms come from adults,[41] we obtained institutional review board permission to recruit age- and gender-matched normal individuals age ≥6 years to provide controls for pediatric study. Respondents with potentially neuropathic conditions were excluded, and those >18 years old underwent 2-hour oral glucose tolerance testing and were excluded for any abnormality. We used standard diagnostic methods, equipment (WR Medical Electronics, Stillwater, MN), and interpretations.[12,41] We measured heart rate variability during deep breathing (6 breaths per minute while supine) and the Valsalva maneuver, hemodynamic responses during 80° head-up tilt, and acetylcholine-evoked sweating. Manufacturer-supplied reference ranges (RRs) defined normality of Valsalva responses. For heart rate variability, values <2.5th centile defined abnormal. For tilt testing, abnormal was a systolic blood pressure drop >20 mm Hg, diastolic blood pressure drop >10 mm Hg,[42] and/or heart rate increase ≥40 (≤18 years) or ≥30 beats per minute (>18 years) within 3 minutes of tilt.[43] For sweat testing, abnormality was defined as a value outside the 95% confidence interval of site-specific, gender- and age-adjusted norms.[41] Performance of AFT caused no adverse events.

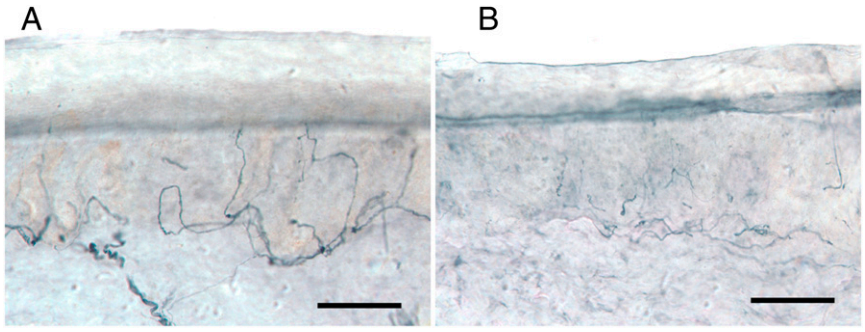

FIGURE 1
Loss of PGP 9.5 immunolabeled nerve fibers in vertical sections from distal leg skin biopsy. A, Biopsy from a 19-year-old white male healthy control contains abundant innervation (675 ENF/mm^2 skin surface area). B, Biopsy from a 19-year-old white male patient demonstrates reduced epidermal (155 ENF/mm^2) and dermal nerve fibers. SFPN was confirmed by skin biopsy; ENF density = 1.3th percentile of laboratory norms. Bars represent 50 μm.

Statistical Analysis

Descriptive statistics, presented as means ± SDs, were used to compare attributes between groups. The χ^2 statistic assessed between-group differences in proportions of abnormal results. Four-site sweat production was compared between patients and controls by using 2-tailed t tests with multiple-sample correction for type I error assuming α = 0.05 and r = 0.5; thus, P < .025 defined significance.

RESULTS

Forty-one consecutive patients were eligible and were included. Mean age at symptom onset was 12.3 ± 5.7 years; age at presentation averaged 20.8 ± 9.1 years. Demographic characteristics were notable for female predominance (73%; P = .001) and ethnic and geographic diversity. Three children lived in Ecuador, Switzerland, and Trinidad, and one had developed symptoms before adoption from Estonia at age 6. Among American-born patients, 1 had 2 Korean parents, and 1 was half Lebanese and half white-American.

Outcome of Diagnostic Testing for SFPN

Overall, 59% (24/41) of patients met the criteria for definite SFPN, although none had undergone all of the 3 tests

analyzed. Specifically, 30% (11/37) of skin biopsies, 100% (2/2) of nerve biopsies, and 53% (18/34) of AFT were definitively diagnostic for SFPN. Minor abnormalities reported in 81% of the remaining skin biopsies (22/26) and 93% of the remaining AFT (14/15) identified an additional 17% of patients with probable SFPN and 22% with possible SFPN. Only 1 of 41 patients had entirely normal results. Two of 2 nerve and muscle biopsies diagnosed SFPN based on Schwann cell stacks that were empty or contained isolated regenerating axons. The biopsied nerves lacked demyelination, loss of large fibers, inflammatory infiltrates, vasculitis, or amyloid. One muscle was normal, and the other had neuropathic and disuse changes.

Comparing AFT results between patients and normal controls revealed 27% of patients (vs 3% of controls; $P < .001$) with reduced heart rate variability during deep breathing, 42% of patients with abnormal cardiovascular responses to Valsalva (vs. 0% of controls; $P < .001$), and 75% with abnormal tilt table results (vs. 18% of controls; $P < .001$). Sweat production (Fig 2B), considered the most sensitive among the autonomic-function tests for diagnosing SFPN,[44] was reduced at one or more among the 4 sites tested in 82% of patients (vs 34% of controls; $P < .001$).

Characterization of Medical Histories, Examinations, and Other Testing

Most patients were moderately or severely ill, explaining their extensive

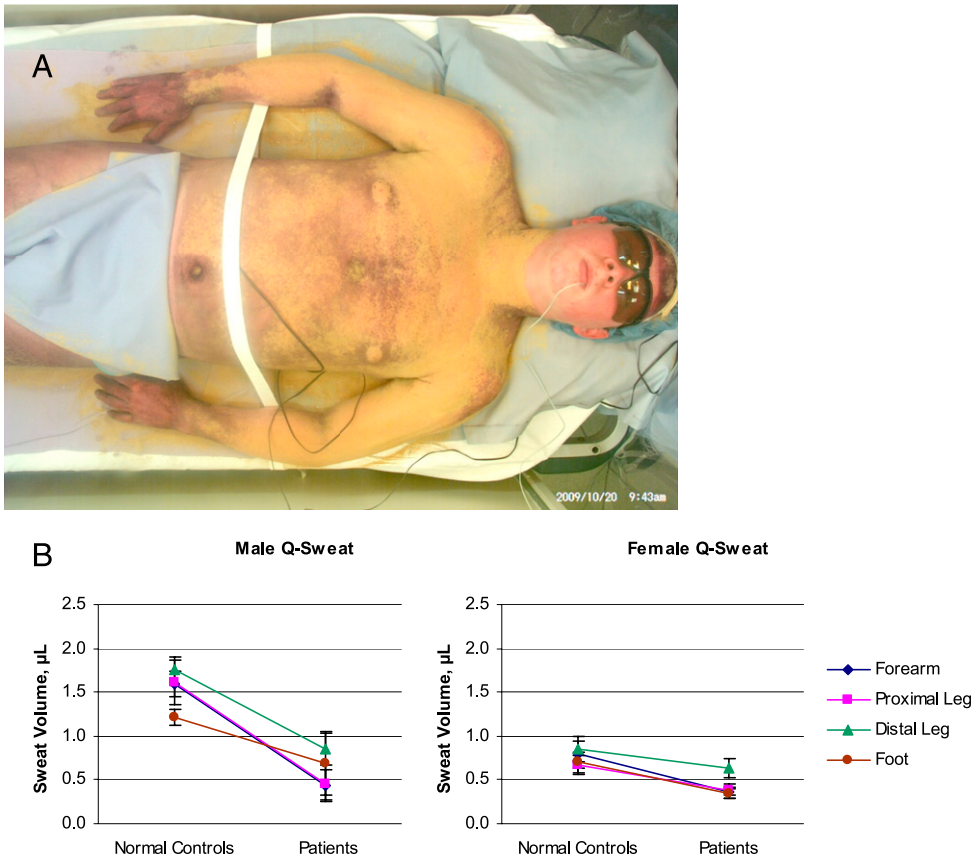

FIGURE 2

A, Patient with delayed sweating on torso and arms during thermoregulatory sweat testing. This 20 year old had non–length-dependent erythromelalgia (pain worsened by heat, plus redness and swelling) that was worse in his cheeks and ears but also affected his hands and feet, labile heart rate and blood pressure, and diarrhea. His distal leg skin biopsy had minor abnormalities, and antinuclear antibodies were present at a titer of 1:80. Thermoregulatory sweat testing involves applying Alizarin red indicator to the skin before controlled heating; it is orange when dry and turns purple when wet. When performed at the Mayo Clinic this revealed delayed sweating on the torso and arms compared with the hands and thighs. This patient's AFT results were normal at the Mayo Clinic but abnormal at Cleveland Clinic and at the National Institutes of Health, with reduced sweating at forearm and distal leg study sites. His patchy/proximal symptoms and test results were consistent with non–length-dependent neuronopathy/ganglionopathy. Image courtesy of Paola Sandroni, MD, PhD, Mayo Clinic. B, Gender- and site-specific comparison of acetylcholine-evoked sweat production in normal controls and patients. All values from patients (n = 33, 10 boys and 23 girls) were compared with all values from gender- and age range–matched controls (n = 38; 19 boys and 19 girls). Symbols depict mean sweat volumes at each study site ± SEM; lines connect same-site groups of controls and patients. Sweating was reduced in male patients versus male controls at the forearm (P = .0011) and proximal leg (P = .0017). Sweating was reduced in female patients versus female controls at the forearm (P = .024), proximal leg (P = .024), and foot (P = .009).

medical evaluations at leading academic medical centers. Sixty-eight percent had been hospitalized, and 68% had required leaves from school or work. No other pathogenic diagnoses explaining their widespread pain (eg, arthritis, myopathy) had been identified. The most common syndromic label at study entry was fibromyalgia; others included functional disorder, central sensitization, pain-amplification syndrome, chronic fatigue, myofascial pain syndrome, and seronegative chronic Lyme disease. Organ-specific syndromic labels included POTS, irritable bowel, functional dyspepsia, abdominal migraine, and chronic daily headache. Four patients also had polycystic ovarian syndrome, and 3 had Ehlers-Danlos syndrome. Psychiatric diagnoses (somatization, conversion) were often considered, although only 1 psychiatric illness had been documented, specifically major depression attributed to unremitting pain.

General examinations consistently identified only sinus tachycardia and abnormal blood pressures, usually orthostatic hypotension. Several patients documented episodic neurogenic blisters. These were often on the limbs, also intraorally and on the face. Occasional patients had sluggish pupillary reflexes.

Most tests other than for neuropathy were noncontributory. Cardiac monitoring revealed sinus tachycardia (5/5) with occasional intermittent bradycardia (consistent with neuropathic dysautonomia), and echocardiography was noncontributory. Electromyography (n = 20) was noncontributory, with only upper-limb denervation identified in 1 patient studied during brachial plexitis. Nerve conduction studies (n = 24) were noncontributory, with polyneuropathy identified in only 2 patients: one with long-standing type 1 diabetes and the other with renal failure from Goodpasture's syndrome. Five patients had borderline or isolated sensory abnormalities that might represent subclinical large-fiber involvement. Gastrointestinal endoscopy was universally futile, but abdominal imaging and motility studies (ie, gastric emptying and Sitz marker studies) sometimes showed slowed motility consistent with neurogenic dysautonomia. Brain imaging by magnetic resonance (n = 23) and computerized axial tomography (n = 8) was nondiagnostic except in 1 patient with fatal hypertensive brain edema and hemorrhage associated with Goodpasture's syndrome. Spinal imaging (n = 22) was noncontributory except for occasionally identifying obstipation. Sleep studies and electroencephalography were futile.

Evaluation for Causes of SFPN

Extensive evaluation for known causes of SFPN identified only 3 potentially causal or contributory systemic diagnoses: 2 patients had Sjögren's spectrum disorders and 1 had type 1 diabetes.[37] The diabetes was proven not to be the cause of this patient's severe SFPN when she had immediate dramatic improvement after corticosteroid administration followed by sustained remission after intravenous immune globulin (IVIG) treatments. Patient histories were overall significant only for prevalence of autoimmune illnesses in 33%. These were usually organ specific and often autoantibody associated, including 6 patients with histories of autoimmune thyroiditis and 2 with Henoch-Schönlein purpura. One each had had episodes of brachial plexitis, type 1 diabetes, postviral arthritis, immune thrombocytopenic purpura, Crohn's disease, autoimmune trochleitis, and Hashimoto's encephalopathy. These, along with family histories of autoimmunity in 52%, suggested the possibility of dysimmune causes of juvenile-onset SFPN. There were no family histories of SFPN, although 1 patient had multiple family members labeled with fibromyalgia who subsequently received research diagnoses of SFPN from our laboratory, consistent with unrecognized familial SFPN.[31]

Sixty-one percent of patients/families attributed onset of their illness to preceding infections or injuries. Twenty-five percent had documented preceding infections including *Mycoplasma pneumoniae*, *Bordatella pertussis*, *Mycobacterium tuberculosis*, group A *Streptococcus*, mononucleosis, influenza, and parvovirus. Among the 11 documented injuries shortly preceding onset of widespread pain, 10 were limb injuries (eg, fractures, sprains, frostbite) featuring excess pain and swelling interpreted as complex regional pain syndrome. These had largely resolved and were distinct from the later widespread and multifocal pain illnesses studied here.

Among patients' extensive body fluid testing (Tables 1 and 2), the only consistent abnormalities were immune related: elevated erythrocyte sedimentation rates (ESRs), antinuclear antibodies, and low levels of complement components C3 and C4. Overall, 89% of patients had $\geq$1 of these abnormalities. A 26% prevalence of high C-reactive protein was considered too nonspecific to analyze further. Type III cryoglobulinemia (IgG, IgM, and C3) without hepatitis was identified in 1 patient (5%) along with profound hypocomplementemia and high ESRs (Fig 3). These abnormalities resolved after treatment with prednisone. Scattered patients had been tested for autoantibodies associated with somatic/painful small-fiber polyneuropathies (peripherin, voltage-gated potassium channel complex, and N-type calcium channels)[45–47]: 1 of 15 tested had peripherin autoantibodies, 1 of 7 had voltage-gated potassium channel complex autoantibodies, and 1 of 11

TABLE 1 Summary of Consistently Abnormal Medical Findings

	Percent (number)
Most common symptoms	
Pain began or was predominant in legs	76% (31/41)
Chronic disabling headaches	63% (25/40)
Chronic disabling fatigue	83% (24/29)
Any autonomic symptom	98% (40/41)
Cardiovascular complaints	90% (37/41)
Gastrointestinal complaints	82% (32/39)
Sweating complaints	63% (22/35)
Urological complaints	34% (11/32)
All consistent abnormalities on neurologic examination	
Erythromelalgia (erythermalgia)	23% (9/40)
Mild weakness of fingers or toes	10% (4/41)
Dyscoordination of fingers or toes	10% (4/41)
Sensory abnormalities	68% (26/38)
Reduced distal cool sensation	68% (25/37)
Reduced distal pinprick sensation	61% (23/38)
Abnormal touch sensation (including allodynia)	34% (14/41)
Reduced distal vibration sensation	21% (8/38)
Reduced distal position sensation	8% (3/38)
Reduced distal reflexes	18% (7/40)
All consistently abnormal blood test results	
Elevated ESR (≥15 mm/h)	37% (15/41)
Elevated antinuclear antibodies (≥1:80 dilution)	45% (17/38)
Reduced C3 (<85 mg/dL)	21% (6/29)
Reduced C4 (<20 mg/dL)	46% (13/28)
Any 1 or more of the above blood test abnormalities	89% (32/36)

had N-type calcium channel autoantibodies. Autoantibodies associated with large-fiber polyneuropathies and pure autonomic polyneuropathies (ie, ganglionic acetylcholine receptor autoantibody) were absent. Two patients' skin biopsies underwent external dermatopathologic analysis including direct immunofluorescence (Fig 3). Both contained significant deposits of C4d, C5b-9, and IgM without inflammatory infiltrates; one also had IgA deposition.

Summary of Treatments for Symptoms of Neuropathic Dysautonomia and Pain

Diagnosing SFPN guided treatment of the patients' symptoms. Hypotension was managed by advising more salt and fluid intake, avoiding rapid or prolonged standing, contracting leg muscles while standing, elevating the head of the bed, and avoiding potentiating medications. Refractory hypotension was treated with compressive stockings and abdominal binders, midodrine, or secondarily, fludrocortisone.[48] Chronic tachycardia was generally well tolerated but if symptomatic was treated with calcium-channel blockers or β-blockers, taking care not to worsen hypotension. Gastrointestinal complaints were addressed with high-fiber diets, small meals, elevating the head of the bed, and avoiding postprandial recumbency. Constipation and nausea/vomiting usually required treatment with medications, and 3 patients required hospital admission for fecal disimpaction. For disabling CWP, gabapentin was most often prescribed, and secondary amine tricyclics (nortriptyline and desipramine) were considered after dysautonomia was excluded or controlled.[49] Opioids were prescribed rarely for severe uncontrolled pain. The erythromelalgia phenotype prompted consideration of mexiletine.

TABLE 2 Summary of Consistently Normal/Negative/Noncontributory Test Results

Cerebrospinal fluid tests:	All tests were normal in 11 patients
Blood tests:	Complete blood count, electrolytes including glucose, renal, liver, and thyroid function, hemoglobin A1c levels, lipids, vitamins, and immunoglobulins, serum protein immunofixation
Urine tests:	Heavy metals, protein immunofixation, porphyrins, amino and organic acids
Infectious tests:	Hepatitis C, syphilis, human immunodeficiency virus, deer-associated zoonotic infections including Lyme, babesiosis, and human monocytic ehrlichiosis
Immune tests:	Rheumatoid factor antibody, Sjögren's autoantibodies (Ro/SS-A, La/SS-B), lupus autoantibodies (anti-dsDNA, Sm, RNP), antineutrophil cytoplasmic antibodies, total complement
Genetic tests:	Only occasionally performed: all tests for genetic neuropathy including Charcot-Marie-Tooth, Fabry, transthyretin, hereditary neuropathy with liability to pressure palsy, also familial hemiplegic migraine, cystic fibrosis

Summary of Treatments With Immune-Modulating Therapies

Immunomodulation was considered for patients with disabling symptoms that were refractory to conservative management. Overall, 80% (12/15) among patients treated with corticosteroids and/or immune globulin improved. Lacking guidelines, doses were extrapolated from childhood GBS or CIDP and case reports of autoimmune SFPN.[21,22,50,51] Short-term daily corticosteroids were considered first on the basis of low cost, wide availability, oral dosing, and low risk of several complications in young patients. Corticosteroids (effective for CIDP but not GBS[52]) were associated with documented sustained improvement in 67% (10/15) of patients and were associated with 1 major adverse event; the diabetic patient developed cataracts.

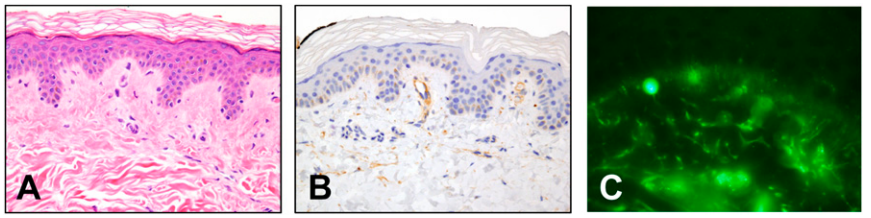

FIGURE 3

This 19 year old had widespread pain, tachycardia, orthostatic hypotension, headache, nausea, and obstipation since childhood. Blood tests identified type III cryoglobulinemia (IgG, IgM, C3), no hepatitis, low complement (C4 = 6, 7, 11; RR, 16–38 mg/dL; C3 = 35, 52; RR, 86–184 mg/dL; ESR = 20–26; RR, 0–17 mm/hour). SFPN was confirmed by skin biopsy; ENF density = 2.4th centile (not shown). Photomicrographs courtesy of Cynthia Magro, MD, Weill Cornell Medical College (vertical sections, 4 μm; 40× magnification). A, Hematoxylin and eosin staining depicting endothelial cell swelling and basement membrane thickening indicating chronic insidious microvasculopathy. B, Immunohistochemical reactivity of dermal microvessels for C4d, a stable component of classic complement activation and a likely correlate of her low serum C4. C, Direct immunofluorescent labeling of IgM deposits on dermal microvessels, a nonspecific marker of microvasculopathy consistent with deposition of the IgM immune complexes present in her blood.

Subsequent tapering of her prednisone caused a relapse of SFPN, so she was transitioned to IVIG with gradual sustained recovery as documented below. Among 5 severely ill hospitalized patients treated with intravenous methylprednisolone (1 g/day for 5 days) followed by a prednisone taper, the 2 acute cases (<3 months) including the index case[22] had rapid, continuing, and objectively documented improvement (see below), whereas the 3 patients who had been ill for several years did not respond. Among clinic patients treated with oral prednisone (1 mg/kg/day for 4 weeks followed by rapid taper), 8 improved and 2 did not.

IVIG[53] was tried in 11 patients who were unresponsive to corticosteroids or were steroid responsive but required long-duration treatment. Three had insufficient treatment for analysis (≤3 doses of 2 g/kg). Among the 8 treated with the standard regimen for autoimmune polyneuropathy (≥3 times with 2 g/kg/month), 3 (38%) did not respond and discontinued treatment, and 5 (62%) had documented significant improvement and continued treatment. Typical infusion-related symptoms responded to standard treatments. One significant adverse event (rash + deep vein thrombosis) resolved. One patient relapsed after IVIG taper and required a few additional treatments.

Objective testing was repeated to monitor treatment efficacy. Six of 6 repeat AFTs among patients treated with immunomodulatory therapies documented improvement, whereas 2 of 2 repeat AFTs in nonimmunomodulated patients did not improve. Two immunomodulated patients studied 3 times each had improved tilt table and sweating responses and heart rate variability during respiration (13.6 → 14.0 → 19.1 and 4.0 → 9.2 → 14.7 beats per minute, respectively). The 1 immunomodulated patient with a low Valsalva ratio at onset normalized (1.42 → 1.76 → 2.27). Four of 4 repeat skin biopsies among immunomodulated patients documented axonal regeneration, which lagged behind symptom improvement. The index patient with 0 ENF/mm^2 skin surface area before corticosteroid treatment had 278 ENFs 4 years later.[22] A 10 year old with 51 ENF/mm^2 skin surface area before corticosteroid and then IVIG treatment had 226 ENFs 14 months later. The 19-year-old diabetic patient with 0 ENFs at baseline had 48 2 years later, after IVIG produced near-total resolution. In the fourth patient, 4 biopsies over 2.5 years corroborated corticosteroid inefficacy followed by IVIG efficacy.

DISCUSSION

This case series provides new hypotheses about childhood-onset, unexplained, acquired CWP syndromes, implicating acquired SFPN, a biologically plausible diagnosis not previously recognized in children. The data suggest that SFPN can develop even in preschool-age children and that juvenile-onset SFPN can persist for decades into adulthood. The characteristic temporal (acute/fulminant versus chronic) and spatial (distal versus patchy/proximal) patterns characteristic of polyneuropathies were all identified.[6] With ubiquitous dysautonomic symptoms, signs, and test abnormalities, juvenile SFPN integrates autonomic as well as neuropathic pain components. Unexpectedly, 63% of patients suffered chronic headaches. These could not be attributed to medication overuse, as few patients used any pain or headache medications. Chronic headache is not currently linked to hypotension, microvasculopathy, or polyneuropathy,[54] but normalizing blood pressure sometimes resolved patients' headaches, consistent with hemodynamic causality.[55,56] The current study has the inherent limitation of case series from academic practices, namely referral bias, which likely elevated the prevalence of SFPN. This limitation should be addressed by prospective reevaluation of these retrospective findings in other settings.

These families' relentless testing for treatable causes of their children's chronic pain generated the abundant data analyzed here. The current results may help to curtail unnecessary testing in other similar children. Imaging was futile, and body fluid testing excluded all common causes of adult-onset SFPN (Table 2).[37,57,58] Laboratory

testing implicated only specific serological markers associated with organ-specific dysimmunity, a known cause of large-fiber polyneuropathies. This study confirms and extends a 32-case series of pediatric erythromelalgia documenting similar results, namely SFPN in 59% of patients and antinuclear antibodies in 46%.[27] That study did not measure complement and did not find high ESRs, conservatively defined as ≥30 mm/hour (Mark Davis, personal communication).[27]

In our study, the most informative serology was low C4 complement in 46% and low-normal levels in most other patients, consistent with innate and autoantibody-mediated immunity. This most likely involves the classic or lectin rather than the alternative complement pathway. Patients' predominantly antibody-associated comorbid autoimmune illnesses and lack of cellular infiltrates within nerve biopsies also argued against T-cell involvement, although cellularity at onset cannot be excluded because nerve biopsies had been performed late in the disease course. Dermatopathological identification of complement (including C4) and immunoglobulin deposition without cellularity in 2 of 2 skin biopsies (Fig 3) provides additional evidence of a role for complement- and autoantibody-mediated dysimmunity in the pathogenesis of some cases of juvenile-onset SFPN.

Sixty-one percent of patients reported specific illness triggers including infections, which are well-known precipitants of organ-specific autoimmunity including neuropathy.[14,59] Thirty-one percent reported a traumatic antecedent. Injury had only rarely been associated with autoimmune neuropathy[60] until the recent seminal discovery of postsurgical autoimmune neuropathies.[61] Most antecedent injuries among our patients initially caused focal pain syndromes labeled as complex regional pain syndrome, which has also been linked to small-fiber neuropathy and autoimmunity.[62–64]

CONCLUSIONS

This study analyzed the results of multiple objective tests to identify a potentially common cause for childhood-onset CWP syndromes: SFPN. It extends the range of SFPN into earliest childhood and characterizes the pediatric presentation. It offers preliminary evidence of disordered immunity in some patients, including hypocomplementemia among other serologic abnormalities, and responsiveness to corticosteroid and immunoglobulin therapy in some patients. Recognition of juvenile-onset SFPN should help patients, families, and clinicians by suggesting a rational pathway for diagnostic evaluation and treatment. Having a specific diagnosis to test for and treat when present may reduce ineffective, costly, and potentially harmful tests and treatments and permit objective testing and definitive treatment of some patients. Additionally, the results demonstrate the need for pediatric norms for tests of SFPN, and they provide new testable hypotheses for clinical and basic research study.

ACKNOWLEDGMENTS

We thank the patients, families, and physicians for providing records; Heather Downs, BS, and Daniela Herzog, BA, for assistance with the normal subjects; Vanda Lennon, MD, PhD, for testing sera for autoantibodies; and Isabelle Rapin, MD, for helpful discussion. This work is dedicated to the memory of John W. Griffin, MD.

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